Therapeutic Strategies for Neurodegenerative Disorders

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Chapter 1

General Introduction

1.1 INTRODUCTION

1.1.1 Neurodegenerative Disorders

Neurodegeneration is considered to be a specific pathological condition in which cells are damaged or cell loss occurs in the central nervous system (CNS). Dependent on the affected cell types, functions, and regions in the CNS, cell impairment or loss could result in distinct physiological, behavioral and cognitive dysfunctions. Neurodegenerative disorders include Alzheimer's disease (AD), brain ischemia, as well as multiple sclerosis (MS) and Parkinson's disease (PD). The causes of neurodegenerative disorders, however, remain still poorly understood. Currently, neurodegenerative disorders affect increasing populations, leading to serious cognitive deficits and even death, which demands effective and affordable diagnostic methods and therapeutic solutions. Although neuroscientists and neurologists have focused on these diseases for decades, the prospects of treating neurodegeneration are still limited.

Alzheimer's disease (AD) is a major neurodegenerative disorder that was first described in 1906 by the German physician Alois Alzheimer. AD is a progressive neurodegenerative disease and the most common cause of dementia related with ageing. The estimation is that AD affected about 26.6 million people by 2006, and the prevalence will quadruple by 2050 (Brookmeyer et al., 2007). This prediction shows that AD will become a global epidemic that will greatly harm people's quality of life. Oskar Fischer (1907) described the physiological histopathology of AD, including that loss of neurons, accumulation of extracellular amyloid-beta (Aβ) plaques, and intracellular neurofibrillary tangles. These histopathological features are mainly observed in hippocampus and cerebral cortex, which play a pivotal role in cognitive functions such as learning and memory. The clinical characteristics of AD patients include cognitive decline, personality changes and memory loss. Despite a great amount of studies on AD, the precise pathological processes underlying this disease remain elusive with various hallmarks including Aβ plaques, tangle formation, and neuronal cell death.

1.1.2 Epidemiology and Genetics in Alzheimer's Disease

Epidemiological analyses of AD patients indicate that almost all AD cases are sporadic and only 1% of AD patients are of familial nature. However, both show the consistent pathology, which suggests a common underlying pathological mechanism of AD. To identify candidate genes that mediate the AD pathological features, families with autosomal dominant forms of AD have been screened for genes that carry mutations. So far, these screenings have revealed three genes to cause familiar AD (Guerreiro et al., 2012), including the gene for amyloid precursor protein (APP) on chromosome 21, presenilin-1 (PS1) on chromosome 14, and presenilin-2 (PS2) on chromosome 1. Mutations in APP gene lead to the development of early onset of AD by increasing the proteolytic cleavage of APP, most of which are linked to the mutations in the PS1 and PS2 genes (Guerreiro et al., 2012; St George-Hyslop and Petit, 2005). Moreover,

apolipoprotein E (ApoE) ε4 is one of the strongest genetic risk factors for the development of AD, and this polymorphic gene is located on chromosome 19, coding for three common isoforms that distinguish at the positions 112 and 158 (Zannis et al., 1982). Even though the precise mechanisms of ApoE's involvement in the pathogenesis of AD remains not completely clear, specific ApoE genotype increases the accumulation of Aβ and is believed to be a main genetic risk factor in both human AD patients and mouse models with AD (Castellano et al., 2011; Rebeck et al., 1993).

1.2 MOLECULAR MECHANISMS UNDERLYING ALZHEIMER'S DISEASE

1.2.1 Amyloid-beta Aggregation

Cerebral Aβ peptides are generated by a series of proteolytic cleavages of the amyloid precursor proteins (APP) via β- or γ-secretases (Selkoe, 1989). APP is present in a number of tissues and organs and probably a trophic factor for neurons (Neve et al., 2000), but APP cleavage products are strictly modulated by neuronal activities (Kamenetz et al., 2003). Aggregation of Aβ from physiological and amyloidogenic cleavage of APP are associated with the central physiopathological events of AD (Hardy and Selkoe, 2002; Zhang et al., 2012b). APP mutations that lead to excessive aggregations of soluble APP peptides are major contributors of amyloid plaques (Citron, 2010). Aβ1-40 and Aβ1-42 plaques are the core components of soluble oligomeric Aβ fibrils to trigger Alzheimer's disease (Balducci et al., 2010; Gandy et al., 2010; Walsh and Selkoe, 2007). Aβ1-42 is more prone to aggregation compared to Aβ1-40 (Selkoe, 1994). The ratio of Aβ1-40 to Aβ1-42, rather than the total amount of Aβ, is an important biomarker for AD development (Duff et al., 1996; Jan et al., 2008; Suzuki et al., 1994). A recent study has also suggested that pyroglutamylated Aβ could be the main APP cleavage product that causes AD because this molecule is not only more cytotoxic than Aβ 1-42 but also more potent in enhancing toxic Aβ (like Aβ3(pE)-42) residue formation (Nussbaum et al., 2012). Furthermore, Aβ1-16 has been demonstrated to be present at much higher concentration in AD patients therefore could serve as a novel marker for the pathogenesis of AD (Portelius et al., 2012). On the other hand, it is worth noting that although Aβ is thought to be a key factor in the pathogenesis of AD, a lower concentration of Aβ protects neurons against oxidative stress (Obrenovich et al., 2002) and only higher concentration of Aβ induces neurotoxicity and disorder of Ca^{2+} homeostasis (Selkoe, 2001).

Additionally, Aβ-induced activation of NMDA receptors causes excitotoxicity and cellular constituent damage. The neurotoxic effects of glutamate are enhanced by introduction of APP cDNA (Ferreira et al., 2010; Tominaga-Yoshino et al., 2001). Studies have also suggested that Aβ induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss (Talantova et al., 2013). The genetic deletion of metabotropic glutamate receptor 5 has been shown to decrease Aβ plaques in a mouse model of AD (Hamilton et al., 2014). Thus, how accumulation of diverse sAPP fragments trigger

neuronal and synaptic dysfunction are urgent topics that need to be resolved in future studies. Evidence has suggested that activated TNF/TNFR1 signalling pathway increases Aβ1-42-mediated toxicity (Shen et al., 1997), while TNFR2 signalling pathway protects neurons against glutamate-induced excitotoxicity (Tominaga-Yoshino et al., 2001). In light of these findings, activating TNFR2 signalling or inhibiting TNFR1 signalling could be a potential approach to treat AD.

1.2.2 Tau Protein

Tau is a highly soluble microtubule-associated protein that is present in axons of healthy neurons and plays a primary role in microtubule (MT) dynamics and axonal transport processes (Li et al., 2011). Tau binds to and stabilizes microtubules and promotes microtubule polymerization (Cleveland et al., 1977). The insoluble tau protein, on the other hand, alters microtubule stability and fast axonal transport (Lee et al., 1994), and results in toxicity of neurofibrillary tangle through the insoluble aggregation of hyperphosphorylated tau proteins . The abnormal phosphorylated tau does not bind to microtubules but is present in high concentration of insoluble fibrillar inclusions. Tau is proposed to be a dominant element in AD, which composes of intracellular neurofibrillary tangles (Selkoe, 1991). Moreover, dendritic function of tau also increases Aβ toxicity in AD mouse models (Chabrier et al., 2012). Because it is uncertain whether tau assembly is contributing to tau-mediated toxicity in neurodegenerative diseases, it is still of question whether tau accumulation inhibitors constitute a feasible therapeutic solution for AD. Some studies show that molecules stabilizing microtubules are considered to be a therapeutic approach to reduce tau function loss, such as axonal dysfunction and cognitive deficits (Zhang et al., 2012). Even though the molecular mechanisms that regulate tau phosphorylation are complex and currently incompletely understood, previous studies demonstrated that NMDA receptors can modulate tau phosphorylation (Burnouf et al., 2013; Ittner and Götz, 2011; Mondragón-Rodríguez et al., 2012). Thereby a potential therapeutic strategy is to reduce NMDA receptors activity to prevent tau protein toxicity. Furthermore, cognitive and synaptic defects mediated via expression of pro-aggregate full-length tau are reversible after switching off expression of pro- and anti-aggregate human tau (Jeugd et al., 2012). Although pathological tau may play a major role to induce AD, this will not be discussed here. Aβ peptide aggregation is thought to be the main upstream signalling event to stimulate tau hyperphosphorylation. The major topic of this thesis is the investigation of NMDA mediated neurotoxicity, Aβ neuropathology and relevant therapeutic strategies for AD and AD related diseases.

1.2.3 Cholinergic System

Pathological alteration of cholinergic neurons and the reduction of acetylcholine levels in the forebrain are among the earliest pathophysiological events of AD (Whitehouse et al., 1982). Degeneration of acetylcholine-releasing neurons and cortical cholinergic denervation significantly contribute to the deterioration in cognitive function in AD patients (Coyle et al., 1983). The strong correlation between the damage of cholinergic

system and cognitive dysfunctions in AD underlies the so-called cholinergic hypothesis of AD (Bartus et al., 1982), one of the most prevailing theories for the cognitive dysfunction in AD patients. Among different cholinergic neuronal populations in the brain, damage of those localized in magnocellular nucleus basalis (NBM) and their cortical projections is correlated with the deficits of memory and learning functions in AD (Iraizoz et al., 1999). The significant loss of choline acetyltransferase (ChAT)-positive neurons in the magnocellular nucleus and their projections in the frontal cortex was observed in homozygous 3xTg-AD mice (Orta-Salazar et al., 2014), further indicating cholinergic system as a potential major player in AD. On the other hand, acetylcholine esterase (AChE) activity is increased around Aβ plaques (Talesa, 2001). One study has demonstrated that NMDA receptor function is impaired by membrane depolymerisation of Aβ in basal forebrain cholinergic neurons (Gu et al., 2014). The fact that the impairment of cholinergic system involves Aβ-mediated degeneration and Aβ aggravates excitotoxicity in cholinergic neurons implies a correlation between cholinergic system degeneration and amyloid β deposition in AD. Moreover, substantial age- and AD-related alterations in Ca^{2+}-responsive proteins mostly contribute to selective vulnerability of basal forebrain cholinergic neurons (BFCN) to degeneration in AD (Riascos et al., 2014). Although the relationship either between cholinergic system and Aβ or between cholinergic system and Ca^{2+} homeostasis remains unclear, cholinergic dysfunction appears to influence APP metabolism and Aβ production and can be affected by Ca^{2+} imbalance.

1.2.4 Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the brain. In neurodegenerative conditions like ischemia or AD, large quantities of glutamate are often released locally which finally trigger progressive neuronal death and cognitive dysfunction (Greenamyre et al., 1988; Rothman, 1986). This effect is likely a result of continuous activation of glutamate receptors, which leads to ion imbalance, subsequent neuronal cell apoptosis, and eventually the development of neurodegenerative disorders (Choi, 1992; Wroge et al., 2012). Glutamate exerts its functions via two pharmacologically distinct receptor types: ligand gated ion channels (ionotropic receptors), which mediate the influx of cations into post-synaptic cells to induce membrane depolarization and can be divided into three classes: AMPA, N-methyl-D-aspartate (NMDA) and kainate receptors; and G-protein coupled receptors (metabotropic receptors), which respond to glutamate through regulation of second messengers, specifically intracellular Ca^{2+} signalling (Hollmann and Heinemann, 1994).

Activation of ionotropic glutamate receptors leads to influx of sodium and calcium ions that need to be pumped out of the neuron in a process requiring energy (Sibson et al., 1998), therefore increases energy consumption. Neurons are particularly vulnerable to glutamate after energy deprivation (Novelli et al., 1988). The ion imbalance caused by excessive Na^{+} and Ca^{2+} influx through glutamate receptors upon ligand binding also leads to cell physiological abnormalities. The drastic increase of glutamate in the synaptic cleft during brain damage could initiate two detrimental processes, which differ in time-

dependency and ionic characteristics. The first process involves acute swelling of cell bodies and dendrites via the opening of membrane cation channels. The Na^+ influx and passive influx of Cl^- ions precede the cell volume expansion. The second one is a delayed response of neuronal degeneration. Evidence suggests that in vitro the neuronal death is related to the $Ca2^+$ influx, which is mainly mediated by NMDA receptors since NMDA receptors exhibit the highest penetration to Ca^{2+} compared other glutamate receptors and thus play a crucial role in glutamate-induced neurodegenerative processes (Choi, 1992). Given the important role of NMDA receptors in glutamate induced neuronal death, such enrichment could be the reason why pyramidal neurons are susceptible to neurodegeneration and massive loss of pyramidal neurons has been observed in AD (Hynd et al., 2004).

1.2.5 Calcium Homeostasis

Deficits in synaptic function through activated NMDA receptors are linked to AD pathogenesis. A hypothetical mechanism of the excitotoxicity of excessive activation of glutamate receptors is to lead to changes of intracellular Ca^{2+} concentration that will eventually activate a cascade of enzymes that causes neuronal cell death or apoptosis (Lipton, 1999). Healthy neurons utilize strict mechanisms to maintain appropriate Ca^{2+} concentration to exert normal function. It has been suggested that Aβ-RAGE interaction disrupts not only neurons but also tight junctions of blood brain barrier via Ca^{2+} concentration changes in AD (Kook et al., 2012). Furthermore, the binding of Ca^{2+} with Aβ peptide can accelerate amyloid plaque formation (Becerril-Ortega et al., 2014), while Aβ can cause calcium mobilization and impair neuronal calcium homeostasis (Badolato et al., 1995; Mark et al., 1995). Through its carboxy-terminal fragment, APP triggers significant changes in intracellular Ca^{2+} concentration and enhances glutamate-induced excitotoxicity (Kim et al., 2000). Neuronal Ca^{2+} signalling involves an intricate interplay between Ca^{2+} influx across the plasma membrane through voltage-gated Ca^{2+} channels, NMDA receptors, and transient receptor potential channels, and Ca^{2+} release from intracellular Ca^{2+}-stores via inositol triphosphate receptor channels in the endoplasmic reticulum (ER) (Bezprozvanny and Mattson, 2008). Large quantities of Ca^{2+} influx in neurons disturbs Ca^{2+} homeostasis and affects the ER function in neurodegeneration (Salminen et al., 2009; Scheper and Hoozemans, 2009). Furthermore, the correlation between the calcium influx and neuronal toxicity is in line with current models of neurodegeneration in which calcium induced mitochondrial dysfuntion plays a key role in neuronal toxicity (Pivovarova and Andrews, 2010). Recent studies have demonstrated that hypothermia can decrease inflammation (Dietrich et al., 2004), prevent apoptosis (Liou et al., 2003), and reduce the extracellular levels of excitatory neurotransmitters (Ooboshi et al., 2000) in neurodegeneration. Hypothermia has been confirmed to reduce calcium influx mediated via activated NMDA receptors (Phillips et al., 2013). This research results provide a novel therapeutic strategy for neurodegenerative diseases like AD by maintaining Ca^{2+} homeostasis.

Marked changes to intracellular Ca^{2+} signal occur prior to cognitive decline and extensive neuronal death in AD (Szydlowska and Tymianski, 2010). Inhibiting the breakdown of Ca^{2+} homeostasis could be an effective therapeutic strategy of neurodegeneration. Abushik et al. revealed that intracellular Ca^{2+} transient induced by the neurotoxic effect of homocysteine in cultured cortical neurons was mediated by activity of NMDA and mGluR5 receptors (Abushik et al., 2014). This indicates that the neurotoxicity of Ca^{2+} influx is probably determined by the crosstalk between NMDA receptors and mGluR5 receptors. Interestingly, Sattler et al. demonstrated that the source of Ca^{2+} entry, not the Ca^{2+} load, is the master determinant of the neurotoxicity of Ca^{2+} (Sattler et al., 1998). Such source specificity of Ca^{2+} neurotoxicity implies that this neurotoxicity is triggered by distinct signal transduction pathways rather than by a general overactivation of Ca^{2+}-dependent downstream pathways. Researchers have also demonstrated that glutamate induced apoptosis involves intracellular Ca^{2+} transient, accumulation of intracellular oxidative stress, DNA damage depletion of GSH, as well as loss of mitochondrial membrane potential (Tirosh et al., 2000).

1.2.6 Oxidative/Nitrosative Stress

Oxidative/nitrosative stress has been postulated to be a critical factor associated with pathophysiological progression of AD, which, at least in part, contributes to destruction of neurons by Aβ. But underlying mechanisms are still poorly understood. Previous studies have shown that excessive neuronal protein oxidation products, protein carbonyl levels, are mainly observed in those regions of the brain where Aβ aggregation is present (Hensley et al., 1995). Furthermore, the primary site of production of reactive oxygen species (ROS) that generates oxidative stress is in the mitochondria. It has also been shown that glutamate-induced neuronal death in vitro was via elevation of intracellular ROS and mitochondrial dysfunction (Tirosh et al., 2000). Surprisingly, Nguyen et al. suggest that in neurodegeneration not only glutamate excitotoxicity and oxidative stress causes mitochondrial dysfunction, mitochondrial dysfunction conversely induces upregulation of activated NMDA receptors and oxidative stress (Nguyen et al., 2011). A vicious cycle, thereby, is in neurodegenerative diseases that comprise glutamate excitotoxicity, oxidative stress and mitochondrial dysfuntions. Due to high oxygen consumption, the brain tissue is particularly vulnerable to the product of reactive oxygen species that greatly increases oxidative stress.

Endogenous nitric oxide (NO), which is a messenger involved in signal transduction, plays a critical role in diverse physiological progresses. In the CNS, NO can be synthesized by Ca^{2+}-dependent neuronal nitric oxide synthase (nNOS) in response to glutamate receptor activation (Garthwaite et al., 1988). A recent study showed that the neuronal nitrosative stress by NO is caused by activity of extrasynaptic NMDA receptors rather than synaptic NMDA receptors in AD (Molokanova et al., 2014). This indirectly indicates that the NO level might involve in physiopathology of neurodegenerative disorders, such as AD. A number of studies have demonstrated that increased NO levels plays a dominant role in neurotoxicity of neurodegenerative disorders (Dawson et al., 1991; Garthwaite et al., 1988; Nguyen et al., 2011). However, another research group show that the retinal neuronal

death induced by Aβ1-42 or glutamate is not involved in increase of NO production (Oliveira et al., 2011). Additionally, Xie et al. have also suggested that neurotoxicity of Aβ or lippolysaccharide-activated microglia is related not to NO generation but to peroxynitrite production generated by inducible NOS (iNOS) (Xie et al., 2002), and inhibitors of iNOS effectively protects neurons against toxicity associated with Aβ-stimulated microglia (Combs et al., 2001). That underlying mechanisms of NO production in neurotoxicity, therefore, is still controversial.

1.2.7 Neuroinflammation

A substantial neuroinflammation characterized by activated microglia might play a central role in AD. Aβ plaques-activated microglia induce the release of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin(IL)-1β, IL-6, IL-8, chemokines, and reactive oxygen and nitrogen species, which are commonly thought of causing neuronal damage (Fernández et al., 2013; Zaheer et al., 2008;Combs et al., 2001;Tan et al., 1999). While microglia also cause a rise in extracellular glutamate levels (Qin et al., 2006), it has been suggested that TNF-α can be produced in response to microglial activation and directly or indirectly potentiate glutmate-meidated excitotoxicity (Leonoudakis et al., 2008). Additionally, TNF-α can mediate such excitotoxicity by rapid triggering the surface expression of Ca^{2+} permeable-AMPA receptors and NMDA receptors (Leonoudakis et al., 2008). Thus, inflammation is also a major mediator in AD, especially microglial activation.

1.3 NEUROPROTECTIVE SIGNALLING AND POTENTIAL THERAPEUTIC STRATEGIES AGAINST ALZHEIMER'S DISEASE

1.3.1 Tumor Necrosis Factor Alpha Receptor Signalling Involvement in Neurodegenerative Disorders

Cytokines are defined as small soluble proteins secreted by cells, which are involved in a variety of inflammatory and infectious conditions. They are not constitutively expressed but rather transiently induced by specific conditions. Tumor necrosis factor alpha (TNF-α), first discovered in the 1970s, is one of the major cytokines (Carswell et al., 1975). TNF-α is generated mainly by monocyte/macrophage lineage, but also by T lymphocytes, neutrophils, endothelial cells, and neurons. It exists in two forms, transmembrane TNF-α (tmTNF-α) and soluble TNF-α (sTNF-α). tmTNF-α is a 26-kDa type II cell-surface transmembrane protein, which can be cleaved by TNF alpha converting enzyme (TACE/ADAM17) to become a 17-kDa soluble form. Both are biologically active. Since clusters of TNF-α receptors are compulsory to initiate the downstream signalling pathways by forming homo-trimeric receptor molecules, TNF-α is also active as a soluble homo-trimeric molecule of 51 kDa (Grell, 1995). TNF-α is a main coordinator to other cytokines (Probert et al., 1996), showing that reduced TNF-α results in the down-regulation of

expression of IL-6, IL-1 and chemokines. Multiple bioactivities of TNF-α are mainly mediated by its reactions to two distinct transmembrane receptors, TNF receptor 1 (TNFR1, a 55-kDa protein) and TNF receptor 2 (TNFR2, a 75-kDa protein). TNFR1 is expressed in almost all tissues whereas TNFR2 is predominantly expressed at a low level in immune cells and endothelial cells (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). tmTNF-α can bind to both TNFR1 and TNFR2 to promote their respective downstream signalling pathways while sTNF-α almost solely binds to TNFR1 (Grell et al., 1995). TNFR1 activation can trigger fibroblast growth, endothelial cell adhesion, neuroinflammation, and cell death while TNFR2 promotes proliferation of thymocytes and peripheral T cells and inhibition of early hematopoiesis (MacEwan, 2002). Several reports have demonstrated that TNFR1 levels are increased in various CNS diseases (He et al., 2007; Scalzo et al., 2009), and its activation triggers severe inflammation and thereby leads to cell death (Kaczmarek et al., 2013).

Various studies have shown that TNF-α plays an important role in many CNS diseases (Ding et al., 2015; Lee et al., 2015; Meissner et al., 2015; Scalzo et al., 2009). Polymorphisms of TNF-α promoter are associated with AD (Di Bona et al., 2009; Ma et al., 2004). It has also been shown that the levels of TNF-α and TNFR1 are both up-regulated in AD (Alvarez et al., 2007; Bruunsgaard et al., 1999), and overexpression of TNFR1 promotes Aβ-induced cell death (Li et al., 2004). Besides, TNFR1 has been illuminated to be involved in the abnormal Aβ processing and may participate in Aβ plaque formation, neuronal damage and cognitive deficits (He et al., 2007). These suggest that preventing TNF-α/TNFR1 signalling pathways could be an efficient strategy in the treatment of AD. Recently, TNFR2 signalling has been concentrated increasingly. Marchetti et al. (2004) reported that TNFR2 signalling mediates neuroprotection via a PKB/Akt-mediated NF-κB pathway against ischemic reperfusion-induced retinal injury and TNFR1 exerts degeneration in this mouse lesion model. Furthermore, there is evidence that expression of TNFR2 is downregulated in AD patients (Cheng et al., 2010). Deletion of TNFR1 significantly prevents amyloid-β (Aβ) plaque formation and remarkably attenuates learning and memory dysfunctions in AD transgenic mice (He et al., 2007). Additionally, TNF-α is demonstrated to exacerbate Aβ-induced toxicity in neurons through inhibiting TNFR2 signalling (Shen et al., 1997).

1.3.2 Tumor Necrosis Factor Receptor-one Antagonists

TNF-α signalling has long been demonstrated to mediated apoptosis and is a critical player in neurodegenerative disorders, such as AD, MS, ischemia/ischemic stroke, in which the levels of TNF-α are remarkably increased. This suggests that anti-TNF-α reagents could be effective as a therapy of neurodegenerative diseases. Anti-TNF-α reagents were tested in clinical trials of MS until Lenercept was reported to fail (1999). The failure of anti-TNF-α therapeutic might be due to the pleiotropic bioactivities of TNF-α, including pro- and anti-inflammatory functions and neurodegeneration as well as regeneration, via its two receptors TNFR1 and TNFR2 (Madsen et al., 2016; Yang et al., 2011). TNFR1 has been demonstrated to result in pro-inflammation and worsening in neurodegeneration (Yang et

al., 2011). Furthermore, TNFR1 genetic variant has also been reported to be one of reasons of failure of anti-TNF-α therapy in MS (Gregory et al., 2012). However, TNFR2 signalling has been implicated to protect against neurodegeneration (Fontaine et al., 2002; Marchetti et al., 2004). Hence, the downstream signalling pathways of TNF-α should be separated depending on its distinct receptors. Selective manipulation of specific TNFRs that are involved in different downstream signalling pathways could therefore provide a beneficial therapeutic strategy.

One way for specific manipulation of TNF-α downstream pathways is to use pharmacological agents that will only block TNFR1 signalling without altering the activation of TNFR2 signalling pathway. For instance, selective inhibitor of soluble TNF-α has been demonstrated to efficiently prevent acceleration of amyloid plaques in transgenic mice by inactivation of TNF-α/TNFR1 signalling pathway (McAlpine et al., 2009). Moreover, a specific human TNFR1 antagonistic antibody has been found to block TNF-α-induced serious inflammation in vitro (Richter et al., 2013). Williams et al (2014) demonstrated that MS symptoms have been strongly prevented by a TNFR1-selective antagonist. Also, a novel TNFR1 antagonist effectively suppressed arterial inflammation and intimal hyperplasia in mice (Kitagaki et al., 2012). Aoki's group (2007) designed a loop peptide mimic of TNFR1 that can inactivate TNFR1 signalling pathway and found that administration of this molecule strongly ameliorates collagen-induced arthritis. Additionally, inhibition of sTNF-α by XPro-1595 which mainly prevent TNFR1 signalling pathways protected EAE mice from clinical symptoms and improved axon preservation and remyelination (Brambilla et al., 2011; Taoufik et al., 2011). These studies show reagents that can block TNFR1 signalling pathway provide a promising treatment method for certain immune diseases not accompanied by drawbacks. It is of interest to test whether similar beneficial effects can be achieved on treating neurodegenerative diseases.

1.3.3 Tumor Necrosis Factor Receptor-two Agonists

A cell-protection effect of TNFR2 signalling was originally shown in a lung injury model (Ortiz et al., 1999), where knockout mice of both TNFRs developed lung injury induced by silica and bleomycin but an up-regulation of TNFR2 significantly prevents silica and bleomycin-induced lung damage in these double knock-out mice. Besides, TNFR2 null mice show sensitized response to cholesterol-bearing pullulan nanogel-induced resorption compared to wild type mice, suggesting a cell-protective role for TNFR2 (Nagano et al., 2011). Fontaine et al. (2002) reported that TNFR2 activation can protect against ischemic reperfusion-induced retinal neuronal death through PKB/Akt-mediated NF-κB pathway, which is in contrast with the role of TNFR1 signalling in this injury model. Activation of TNFR2 signalling pathway therefore might be as a therapeutic approach against degeneration. Recently, the tests of potential therapeutic effects of TNFR2 pathway in neurodegenerative disorders have recently been reported. Fischer et al. (2011) demonstrated that increase of TNFR2 signalling by its agonist significantly prevents neuronal cell death induced by oxidative stress. Future studies will clarify whether TNFR2 agonists could be developed to be effective drugs in treatment of neurodegenerative diseases, such as the early stage of AD that was markered by cholinergic denervation. This

successful therapeutic might be a novel approach to solve the AD problem at its early stage, and might provide a greater sense of hope for AD patients.

1.3.4 Immunization and Immunotherapies

AD is one of the most common dementia in aged population. Given that there is still no effective therapeutic solution for this disease, it presents an immense challenge to neuroscience research and clinical community to search for a more effective approach for treatment. A number of studies have investigated potential agents in the treatment of AD. For instance, the Wang's group recently found that erythropoietin (EPO) is neuroprotective against AD-induced pathology and cognitive dysfunctions (Li et al., 2015). However, its neuroprotective mechanism remains unclear. Overall, it is difficult to judge whether these molecules applied for AD treatment would simultaneously cause adverse effects. As we know the main pathological hallmarks in AD are Aβ plaques and tau protein-derived neurofibrillary tangles. Therefore, potential therapeutic strategies through inhibiting formation Aβ plaques and/or neurofibrillary tangles should be developed and investigated.

Among different strategies, immunotherapy perhaps represents one of the best solutions because immunization is a chronic therapeutic approach and can be applied prior to the occurrence of symptoms. Because Aβ peptides form extracellular plaques and fibrillary tangles are formed within neuronal cells, increasing efforts have been paid to the investigation of Aβ antibodies. Major efforts are now set on passive immunization with preformed antibodies and active immunizations with epitope vaccines only using the B cell epitope for antibody specificity, avoiding the Aβ42 T cell epitope and therefore a potential T cell response (Carrillo et al., 2013; Lambracht-Washington and Rosenberg, 2013). The passive immunization has been applied for clinical trials, in which patients received Aβ1-42 peptide immunization (Fox et al., 2005). Compared with passive immunotherapy, active immunization is much less costly and can be easily applied to large populations. More interestingly, a research group produced a DNA vaccine, p($A\beta_{3-10}$)-IL-4,encoding ten tandem repeats of Aβ3-10 fused with mouse interleukin-4 as adjuvant to immunize eight-month-old APP/PS1 transgenic mice and found that immunization with this molecule significantly reduces Aβ peptide plaques in the hippocampus and the cortex and attenuates cognitive deficits (Xing et al., 2015). Vaccination against Aβ was considered to be the most promising therapeutic strategy until immunized patients showed that strong brain inflammatory response (Imbimbo, 2002; Münch and Robinson, 2002). Potential safety concerns of anti-Aβ vaccines render the investigation of alternative strategies to reduce Aβ burden in AD. Because soluble oligomeric forms of Aβ are considered to be the major mediators of cytotoxicity in AD, neuroscientists and neurologists increasingly focus on therapeutic strategies that can prevent Aβ to accumulate and/or form oligomers. A recent article showed that adjuvant immunotherapies in the presence of anti-Aβ antibodies can promote Aβ uptake capacity by the activation of peripheral monocytes (Hallé et al., 2015). Alternatively, our group (described in Chapter 6) also tested a novel vaccine, which was derived from Aβ and prevented the toxicity of Aβ1-42 oligomers (Mulder et al., 2016).

Aβ pathology leads to the downstream pathological accumulation of tau. Tau immunotherapy has also been tested in AD. Different studies have shown that active and passive immunization approaches were effective in reducing neurofibrillary tangles in the brain, delaying the onset of motor function decline of tau tangle pathology or slowing the progression of cognitive deficits (Asuni et al., 2007; Boutajangout et al., 2010; Davtyan et al., 2014). Interestingly, tau immunotherapy by using tau oligomer-specific monoclonal antibody reduced Aβ levels, suggesting a link between tau and Aβ oligomers (Castillo-Carranza et al., 2015). These data suggest that immunotherapy could be a promising strategy for treatment of AD.

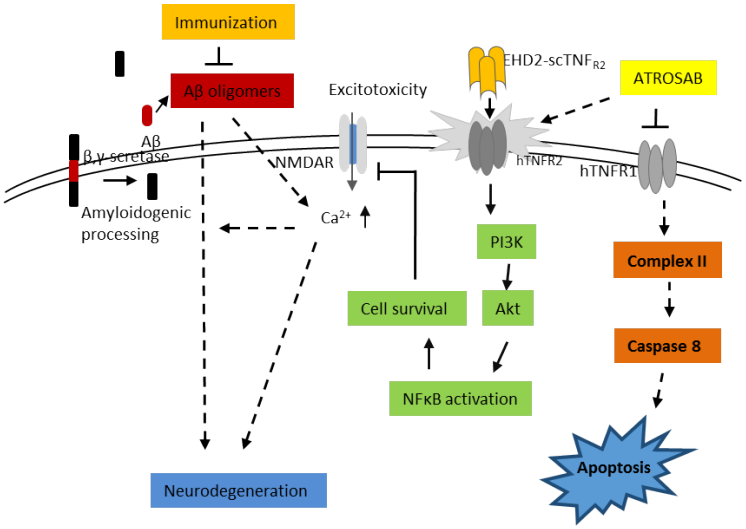

Figure 1.1. Schematic overview of neuroprotective strategies for Alzheimer's disease. Activated TNFR2 can lead to NF-κB activation to initiate transcriptions of cell survival factors and thereby to mediate neuroprotection. TNFR1 antagonists block TNFR1 apoptotic signalling pathway and share TNFR2-mediated neuroprotection. Moreover, anti-Aβ oligomers by immunization inhibit Aβ peptides to form soluble Aβ oligomers that are toxic and induce apoptosis in the brain.

1.4 THE NUCLEUS BASALIS LESION

The nucleus basalis meynert (NBM, known as nucleus basalis magnocellularis in the mouse) is an 'open' nucleus with no distinct boundaries and it forms several clusters within the basal forebrain. The NBM is a major source of cholinergic innervation of the cerebral cortex (Mesulam and Van Hoesen, 1976). The NBM in AD patients shows a substantial reduction of cholinergic neurons (Whitehouse et al., 1981). Also, NBM undergoes a profound degeneration manifested by a loss of cholinergic innervations in the cerebral cortex in the patients with AD and senile dementia of Alzheimer's type and the NBM cholinergic neuronal loss occurs early in the course of the disease (Whitehouse et al.,

1982). These findings suggest that a pivotal reduction of cholinergic neurons and their projections induced by the NBM lesion is a pathological hallmark in the early stage of AD. Furthermore, the NBM lesion-mediated cholinergic denervation rapidly and persistently induced amyloid precursor in the cerebral cortex (Wallace et al., 1993), indicating that loss of NBM cholinergic innervations may also participate in Aβ plaque formation. Importantly, cholinergic neuronal damage in the NBM and the NBM lesion-induced cholinergic denervation in the cortex result in cognitive dysfunctions that mimic cognitive deficits of AD, such as impaired learning performance and memory formation (Bartus et al., 1982; Iraizoz et al., 1999). These neuropathological studies suggest that the basal forebrain cholinergic system is affected in AD. Moreover, the NBM lesion model provides a valuable platform to investigate and evaluate the pharmacological capability of potential drugs (Harkany et al., 1995; Van der Zee et al., 1994). In addition, we can directly identify the lesion by the measurement of the cholinergic fibres in the forebrain cortex. In this thesis, we utilized this lesion model to test the effectiveness of pharmacological agents.

1.5 OUTLINE OF THIS THESIS

Neuronal and synaptic loss ultimately results in functional impairments and even death of patients in AD and related neurodegenerative diseases. A better understanding of the pathogenic mechanisms of AD is critical to develop effective therapeutic solutions to prevent or treat AD. This thesis elaborates the therapeutic strategies in prevention or treatment of AD with respect to TNF-α functions as well as passive immunization. Several studies showed TNF-α duality: it not only enables neuroprotective effects against glutamate-induced excitotoxicity but aggravates the pathological features in neurodegeneration. Although the molecular mechanisms underlying these effects of TNF-α are largely unclear, it has been proposed that TNF-α binding to TNFR2 increases neuronal survival via the activation of PKB/Akt and NF-κB pathways. In contrast, TNFR1 activation mediates degeneration in neurodegenerative diseases. Research in this thesis aims to not only gain information on the underlying molecular etiology of AD development but also identify novel targets for potential therapeutic intervention. TNF signalling is a rather complex process and we therefore mainly focused on TNF-α signalling mechanisms via its receptor 1 and receptor 2 in various neurodegenerative disorders (Chapter 2). Physiological functions of individual TNF-α receptor signalling are briefly described. This chapter provides a general description on TNF-α dual functions in neurodegenerative disorders and predicts differentially targeting TNF-α signalling pathways through its two distinct receptors, could lead to development of effectively therapeutic approaches.

To assess the potential therapeutic effects of human TNFRs targets *in vivo*, such as TNFR1 antagonists and TNFR2 agonists, which have been implicated neuroprotection, we designed and generated humanized TNFR1/2 knock-in mouse models as described in Chapter 3.

Epidemiological studies also suggest that targeting TNFRs could be useful for the prevention of AD (Fischer et al., 2011; McCann et al., 2014; Williams et al., 2014). Thereby one option could be to adjust TNF-α signalling in order to either slow or halt pathological processes associated with AD pathology. For this purpose, research was conducted to study the potential neuroprotective effects of human TNFR2 selective agonists, TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$ and human TNFR1 specific antagonist, ATROSB in humanized TNFR knock-in mice. Their potential neuroprotective effects are investigated in an in vivo model for neurodegeneration in which nucleus basalis magnocellularis (NBM) was lesioned by NMDA in both huTNFR ki mouse lines (Chapter 4).

In chapter 5 it is investigated whether the reagents, ATROSAB and EHD2-scTNF$_{R2}$, can ameliorate behavioral deficits induced by NBM lesion in two mouse strains (hu/mTNFR1-k/imice and hu/mTNFR2-k/i mice) by using tests for a spontaneous alternation, an elevated plus maze, and a passive avoidance task. These tests should give us an indication for the functional relationship with the NBM lesion.

Aβ plaques and tau neurofilament tangles have been confirmed in human AD patients. Therefore preventing the aggregation of Aβ plaques and fibril tangles could be another effective therapeutic strategy. Chapter 6 therefore focuses on the investigation of the prevention of the accumulation of Aβ plaques. A vaccine based on the Aβ derived short synthetic peptides mimicking Aβ1-42 oligomer epitopes was used to immunize mice and tested in the NBM lesions induced by Aβ1-42 oligomers.

Chapter 7 will discuss the major results of this thesis and will provide a short summary.

Overall, this thesis discusses therapeutic strategies based on our current understanding of the AD pathogenesis.

1.6 FUTURE PERSPECTIVES OF AD THERAPEUTICS

A major challenge to study therapeutic strategies of AD is its complex neuropathological mechanisms, including Aβ accumulations, tau hyperphosphorylation, cholinergic system defecits, oxidative stress, neuroinflammation as well as calcium dyshomeostasis. Neuroscientists and neuorologists attempt to search effective therapeutic approaches according to complex neuropathological mechanisms of AD. So far, almost therapeutic strategies related to whether inhibiting amyloid aggregation or preventing tau hyperphosphorylation are failure to treat AD. For instance, the failure of γ- or β-secretase antagonists against AD might be linked to their poor permeability from blood-brain-barrier and the indiscriminate inhibition of other substrate processes and thereby lead to some severe adverse effects (Lee et al., 1998). Moreover, immunization with anti-Aβ vaccines also failed in clinical trials. Thus, neuroscientists and neuorologists have to expend investigations and to search potential therapeutic approaches against AD from other directions.

Several studies have shown that cytokine expression is drastically altered in various neurodegenerative disorders (Bhaskar et al., 2014; Fillit et al., 1991). Since anti-TNF-α agents were widely used to treat inflammatory disease, neuroscientists have studied whether anti-TNF-α agents could be able to therapy neurodegenerative disorders, like AD. However, anti-TNF-α reagents induced some side-effects in patients with multiple sclerosis (Sicotte and Voskuhl, 2001). Studies have shown that up-regulated expression of TNF-α, TNFR1 and TRADD can be observed in AD, whereas TNFR2 and Fas-associated death domain-like interleukin-1-beta converting enzyme inhibition protein (FLIP) is down-regulated in AD and progressive neuronal insult occurs (Fillit et al., 1991; Zhao et al., 2003). Moreover, deletion of TNFR1 in AD mouse models amyloid deposits and attenuated cognitive dysfunctions suggested that activation of TNFR2 is neuroprotective in AD (He et al., 2007). Together those observations suggest a connection between TNF-α and progression of AD: TNF-α duality including both neuron aggregation and neuron survival via the two distinct receptors in AD (Williams et al., 2014, Dong et al., 2016). Therefore, dependent on the opposite roles of TNF-α interacting with two distinct receptors, our group thought that selective targeting TNFRs, inhibiting TNFR1 signalling and activating TNFR2 signalling, could effectively prevent neurodegenerative disorders. We indeed found that both TNFR2 agonist and TNFR1 antagonist prevent the neocortical denervation and improve the related memory deficits (Dong et al., 2016), as described in this thesis. This therapeutic strategy could improve the therapeutic deficits of anti-TNF-α agents because selective inhibiting TNFR1 and/or activating TNFR2 changed the rate of TNFR1/TNFR2 towards neuroprotective signalling and inhibiting TNFR1 rather than suppressed all TNF-α signalling. Developing TNFR1 antagonists and TNFR2 agonists might be a novel access to solve the AD problem.

In future, several research directions might be concentrated to search therapeutic strategies against AD. Targets that can selectively activate TNFR2 signalling pathway and/or inhibit TNFR1 signalling pathway might be developed drugs to treat AD, such as TNFR1 inhibitors and TNFR2 agonists. Some protein accumulations and activations mediate TNFR1-downstream signalling, specifically TREDD and FADD recruitments and activation. Therefore, another therapeutic strategy for AD could be that prevention of TREDD activation or FADD activation depresses TNFR1 death signalling. An advantage of this strategy might be that just prevention of TREDD and FADD inhibits TNFR1-mediated death signalling but not interfere TNFR1-mediated other signalling pathways such as TNFR1-activated NF-κB signalling or TNFR2 neuroprotective effects. In future, some novel therapeutic strategies based on cytokine signalling pathways could be prevalent against neurodegenerative disorders.

Additionally, accumulation of Aβ plaque peptides is responsible for the onset of AD. Although Aβ plaques are involved in the pathology of AD, Aβ plaques also seems to occur in non-diseased aging people (Dugger et al., 2014; Elobeid et al., 2016; Kovacs et al., 2013). As we know, it seems that Aβ oligomers are mainly responsible for toxic stress in the brains in AD patients. Although, inhibiting the accumulation of Aβ plaques will continue to be a main therapeutic strategy against AD in future. In particular, immunization against oligomeric Aβ appears to be promising strategy. However, immunization is a complicated

approach that involves vaccine optimization and the possibility of side-effects. Nevertheless it also seems an important and powerful strategy to prevent Alzheimer's disease.

Because AD is a complex pathological process related many etiologies, neuroscientists and neurologists thoroughly understand the precise mechanisms underlying AD, and then could rolve the problem on treating AD.

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Chapter 2

Targeting of Tumor Necrosis Factor Alpha Receptors as a Therapeutic Strategy for Neurodegenerative Disorders

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ABSTRACT

Numerous studies have revealed the pleiotropic functions of tumor necrosis factor alpha (TNF-α), and have linked it with several neurodegenerative disorders. This review describes the signalling pathways induced by TNF-α via its two receptors (TNFR1 and TNFR2), and their functions in neurodegenerative processes as in Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and ischemic stroke. It has become clear that TNF-α may exert divergent actions in neurodegenerative disorders, including neurodegenerative and neuroprotective effects, which appears to depend on its signalling via either TNFR1 or TNFR2. Specific targeting of these receptors is a promising therapeutic strategy for many disorders.

2.1 INTRODUCTION

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine implicated in multiple inflammatory diseases, including cancer, rheumatoid arthritis, and in neurodegeneration as in Alzheimer's disease (AD), stroke, multiple sclerosis (MS), Parkinson's disease (PD) (Bruce et al., 1996; Franciotta et al., 1989; Lourenco et al., 2013; Moriwaki et al., 2015; Siebert et al., 2015). TNF-α is synthesized as a type II transmembrane protein of 26 kDa and forms a stable homo-trimeric molecule (mTNF-α) to exert its pleiotropic biological activities. It can be processed by proteolytic cleavage via TNF alpha converting enzyme (TACE/ADAM17) to a 17 kDa monomeric protein, that is biologically active as a soluble homo-trimeric molecule of 51 kDa (sTNF-α). TNF-α induces various cellular responses through its interaction with two distinct transmembrane receptors, the 55 kDa TNF receptor type I (TNFR1) and the 75 kDa TNF receptor type II (TNFR2). Under normal physiological conditions, TNFR1 is ubiquitously expressed in various cell types and tissues, whereas TNFR2 is predominantly expressed at low levels in immune cells and endothelial cells (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Cabal-Hierro and Lazo, 2012). TNFR1 activation can be induced by either sTNF-α or mTNF-α, and TNFR2 activation is predominantly initiated by mTNF-α (Grell et al., 1995). TNF-α is classically known to exert pro-apoptotic functions via TNFR1 (Fiers et al., 1995) and via TNFR2 in co-operation with TNFR1 (Weiss et al., 1997, 1998). On the contrary, more recent evidence clearly indicates that TNF-α induces cell survival and cellular proliferation via its TNFR2 (Van Herreweghe et al., 2010; Okamoto et al., 2009). This led to new insights that TNF-α exerts opposing effects via its two receptors with respect to neurodegeneration and neuroprotection. The potent pro-inflammatory functions of TNF-α in the brain play an important role in the etiology of neurodegenerative disorders such as ischemia/ischemic stroke, MS as well as AD (Fontaine et al., 2002; Van Herreweghe et al., 2010; Rossi et al., 2014; Williams et al., 2014). Therefore, a thorough understanding of TNF-α signalling pathways in neurodegenerative disorders can promote the development of effective agents in the treatment of these conditions.

This review will discuss TNF-α-mediated functions in the healthy and unhealthy central nervous system (CNS), focusing on TNF-α mediated signalling pathways via its receptors, TNFR1 and TNFR2. Finally, we will examine these signalling pathways as potential therapeutic strategies for neurodegenerative disorders, focusing on AD, PD, ischemic stroke and MS.

2.2 TNF-ALPHA RECEPTOR SIGNALLING PATHWAYS

2.2.1 TNF-α Receptor One Signalling

The two distinct transmembrane receptors of TNF-α are characterized by a conserved domain of N-terminal repeating cysteine-rich motif in extracellular regions that specifically interact with diverse TNF-related ligands (Locksley et al., 2001). The conserved domain is necessary and sufficient for the preassembly of TNFR complexes that bind TNF-α trimers and mediate downstream signalling (Chan et al., 2000a). However, the intracellular domains of the two receptors lack homologous sequences (Lewis et al., 1991), suggesting that distinct signalling pathways emanate from the two receptors. TNFR1 is distinguished from TNFR2 by its intracellular death domains (DDs) (Tartaglia et al., 1993). The extracellular domain of TNFR1 contains well-ordered cysteine rich amino-terminal, known as the preligand binding assembly domain (PLAD). PLAD favours pre-assembly of TNFR1 into trimeric complexes and functions as preventative measure for receptor auto-activation and is essential for ligand binding (Chan et al., 2000b). TNFR1 can be activated by both sTNF-α and mTNF-α (Grell et al., 1995). TNFR1-induced apoptosis involves formations of two sequential signal complexes (complex I and complex II) that are separated both temporally and spatially, but is limited by the successful activation of complex I (Micheau and Tschopp, 2003a). After TNF-α binding, the activated TNFR1 acts via its intracellular DDs to recruit the core adaptor TNFR-associated death domain (TRADD) (Chen and Goeddel, 2002; Chen et al., 2008; Hsu et al., 1995). Furthermore, receptor-interacting protein 1 (RIP1) is recruited rapidly and modified with non-degradative poly-ubiquitin chains (Meylan et al., 2004; O'Donnell et al., 2007). TRADD subsequently recruits other proteins such as TNFR-associated factor 2 (TRAF2) (Wang et al., 1998a), inhibitor of apoptosis protein 1 (cIAP1) and inhibitor of apoptosis protein 2 (cIAP2) (Varfolomeev et al., 2008a), forming the initial signalling complex I. This signalling complex with RIP1 ubiquitination initiates the later activation of the catalytic IκB kinase (IKK) complex (Ea et al., 2006). The activated IKK complex, which consists of an IKKα subunit and an IKKβ subunit, functions as an essential regulatory subunit of IKK complex (IKKγ/NEMO), which subsequently phosphorylates the inhibitor of kappa-B (IκB) complex (Karin and Ben-Neriah, 2000). The IκB complex is then degraded via the ubiquitin-proteasome. Consequently, NF-κB composed of p50/p52 and RelA (p65)/RelB subunits is released and translocated into the nucleus to initiate transcription of anti-apoptotic genes; cIAP-1, cIAP-2, TRAF1, TFAF2 as well ascellular FLICE-like inhibitory protein (cFLIP), to inhibit caspase 8 release from complex II(Ea et al., 2006; Micheau et al., 2001a; Wang et al., 1998a) (Figure 2.1). The caspase-8 inhibitor cFLIP(L) which is harbored in complex II simultaneously inhibits the release of pre-caspase 8 (Micheau and Tschopp, 2003a; Micheau et al., 2001a).

When complex I signalling fails to activate the NF-κB pathway in some instances, the activated TNFR1 will recruit the complex II to trigger apoptotic processing (Figure 2.1). After ligand binding to TNFR1, silencer of DDs is dissociated from the intracellular domains of TNFR1, and recruits the adaptor proteins such as TRADD, RIP1, TRAF2 and Fas-associated death domain (FADD) and pre-caspase 8 to form complex II (Jiang et al., 1999). In the activated complex, FADD triggers pre-caspase 8 activation, resulting in the release of p18/p12 fragments that can trigger downstream caspase cascades to participate in apoptotic processes (Micheau and Tschopp, 2003a). This pro-apoptotic signalling mechanism involves FADD and caspase 8 as the key factors to trigger apoptosis (Juo et al., 1998; Yeh et al., 1998). Upon recruitment of FADD and caspase-8, initiation of apoptosis

via caspase-8 is mainly determined by levels of the anti-apoptotic protein; cFLIP(L) (Micheau et al., 2001b). NF-κB activation triggered by complex I signalling determines the availability of cFLIP(L) protein at the moment complex II is formed (Micheau et al., 2001b). Therefore, adequate production of cFLIP(L) via NF-κB upon complex I signalling, prevents subsequent caspase-8 mediated apoptosis of the complex II signalling pathway (Micheau and Tschopp, 2003b).

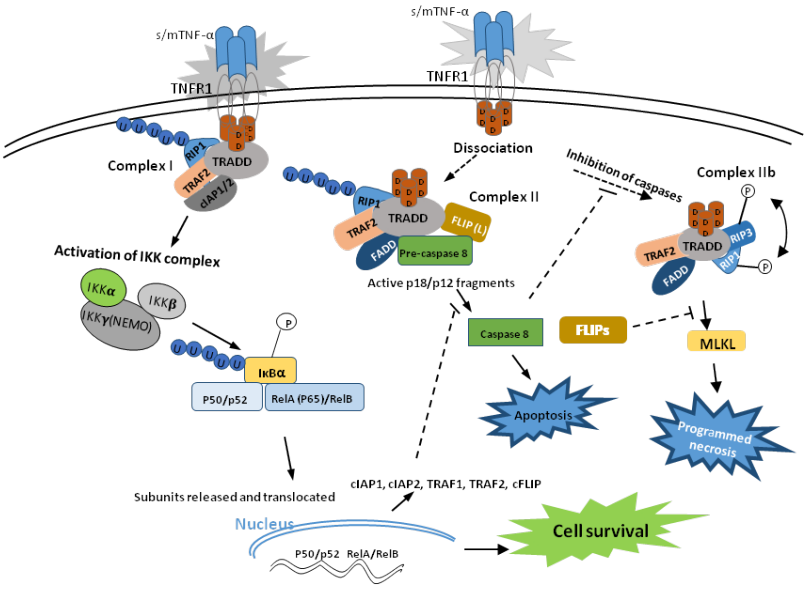

Figure 2.1. Multiple forms of TNFR1 signalling activation are mediated through intracellular protein complex assembly. The trimeric TNF-α engagement of TNFR1 leads to cellular apoptosis or cell survival via the distinct complex signalling pathways.

TNFR1 signalling can also trigger caspase-independent programmed necrosis (necroptosis) in Jurkat cells and in ischemic brain injury (Degterev et al., 2005; Holler et al., 2000; Vercammen et al., 1998). It was shown in mouse embryonic TNFR1$^{-/-}$ fibroblast cells that necrotic cell death is primarily dependent on TNFR1-mediated pathways (Lin et al., 2004). In this process, RIPs, FADD and TRAF2 elements are still the critical components to form complex IIb (Lin et al., 2004) (Figure 2.1). RIP1 is required for the formation of complex IIb (Degterev et al., 2008). Additionally, RIP3 assembly is essential for RIP1 recruitment to this complex and identified as a crucial kinase to phosphorylate RIP1, which in turn phosphorylates RIP3 to form the RIP1-RIP3 pre-necrotic complex (Cho et al., 2009). This activated complex phosphorylates the downstream mixed lineage kinase domain-like protein (MLKL) which subsequently triggers necrosis (Sun et al., 2012). MLKL is therefore a critical factor in RIP3-mediated downstream necroptotic pathways. Dephosphorylation of RIP3 via protein phosphatase 1B (Ppm1b) restricts necroptosis (Chen et al., 2015). In addition to caspase suppression, cFLIP proteins can potentially inhibit TNF-induced necroptosis (Chan et al., 2003). Necroptosis is a newly discovered pathway of cell death

with essential functions in tissue homeostasis and development, and particularly studied in cancers and skin diseases. However, research on this pathway remains rather limited in CNS conditions. In primary hippocampal neurons necroptosis was induced via RIP3 upon an ischemic insult (Vieira et al., 2014). Moreover, it was recently shown *in vivo* in mice that intracerebroventricular injection with TNF-α caused RIP3-mediated necroptosis of hippocampal neurons (Liu et al., 2014). Besides neurons, necroptosis can also occur in activated (by stimulation with different inflammatory stimuli, including TNF-α) primary microglia, upon inhibition of caspase-8 (Fricker et al., 2013). Interestingly, in mixed cultures (where also primary neurons and astrocytes were present), necroptosis of activated microglia protected neurons from cell death (Fricker et al., 2013). Yuan's group (2015) reported that necroptosis in cortical neurons mainly depends on the RIP1-RIP3-MLKL signalling pathway induced via TNF-α/TNFR1 in MS. Przedborski's group (2014), yet, showed that necroptosis-driven death of motor neurons triggered by amyotrophic lateral sclerosis (ALS) involves the RIP1-MLKL signalling pathway independent of TNF signalling. As such, it seems that RIP1-MLKL mediated neuronal necroptosis may be induced in different ways, both are dependent or independent of TNF-α.

2.2.2 TNF-α Receptor Two Signalling

Compared to TNFR1-mediated pathways, TNFR2-mediated signalling is still less well understood. TNFR2 is typically expressed at a low level in cells of the immune system, and is activated primarily by mTNF-α (Grell et al., 1995). Unlike TNFR1, TNFR2 does not include a death domain (DD). Trimerization of TNFR2 is induced upon binding of mTNF-α, leading to the recruitment of TRAF2 to the intracellular TRAF binding motif, which subsequently causes the recruitments of TRAF1, cIAP1 and cIAP2 (Figure 2.2). TRAF2 is a key mediator in this signalling pathway that triggers the subsequent signalling cascades leading to activation of NF-κB (Cabal-Hierro et al., 2014). The activation TRAF2-cIAP1/2 complex is recruited to NF-κB-inducing kinase (NIK) by TRAF3 resulting in proteosomal degradation of NIK (Sun and Ley, 2008). TNFR2 activation by mTNF-α maintains NIK stabilization via induction of TRAF3 degradation and thereby activates IκBα, leading to phosphorylation and activation of IKKα. Phosphorylated IKKα consequently activates the NF-κB precursor protein p100 (Rauert et al., 2010). This noncanonical NF-κB activation is independent of IKKβ and IKKγ (Sun and Ley, 2008). Moreover, it has been reported that human TNFR2 contains a second intracellular binding region for TRAF2 and that this intracellular region can recruit TRAF2, which leads to the activation of NF-κB, dependent on activation of NIK and IκBα. Deletion of this binding region impairs the ability of TNFR2 to activate NF-κB (Rodríguez et al., 2011).

TNFR2-mediated activation of NF-κB can also occur via the phosphoinositide 3-kinases (PI3K) - protein kinase B/serine-threonine kinase (PBK/Akt) signalling pathway (Marchetti et al., 2004) (Figure 2.2). The phosphorylated IκB is degraded via the ubiquitin-proteasome and NF-κB is translocated into nucleus to initiate transcription (Gustin et al., 2004). TNFR2-mediated NF-κB activation is down regulated by phosphatase and tensin homolog deleted on chromosome ten (PTEN), which is a strong inhibitor of PI3K-PBK/Akt pathway. However, NF-κB activation via TNF-α leads to down regulation of PTEN (Eisel et al., 2006).

In addition to NF-κB activation, signalling via TNFR2 can elicit various non-apoptotic responses including c-Jun N-terminal kinase (JNK) or p38 mitogen-activated protein kinases (MAPK) that depends on the recruitment of TRAF2 to the different intracellular binding sites of TNFR2 (Matsuzawa et al., 2008; Ruspi et al., 2014). In these signalling pathways, TNFR2 binds TRAF2 to its intracellular region, and subsequently recruits TRAF1, TRAF3, cIAP1 and cIAP2 (Rothe et al., 1995; Varfolomeev et al., 2008b). According to spatial and temporal separation, this complex binds to a MAPK kinase kinase (MAP3K) protein called MEKK1 and enhances the activity of this kinase and phosphorylation of JNK-activating kinase (JNKK1) (Baud et al., 1999), and thereby stimulates JNK activation to promote downstream signalling pathways which mediate cell survival. It should be noted however that while acute activation of JNK via TRAF2 has been related with cell survival, prolonged activation may also lead to apoptosis (Chen and Tan, 2000; Dhanasekaran and Reddy, 2008; Tabas and Ron, 2011). TNF-α mediated JNK activation depends, at least in part, on MEKK1 (Baud et al., 1999; Matsuzawa et al., 2008). As opposed to TRAF1 and TRAF3, TRAF2 positively regulates JNK activation (Cabal-Hierro et al., 2014). It has been suggested that TNFR2 harbours two sequences that are adaptors specific for JNK signalling (Ji et al., 2012). TNFR2-TRAFs/cIAPs can mediate activation of p38 MAPK signalling pathway. TRAF2 is a major player to promote activation of p38 MAPK signalling pathway (Cannons et al., 2000). In this pathway, the TRAFs/cIAPs complex recruits RIP and subsequently activates MKK3 to initiate p38 MAPK activation (Aggarwal, 2003; Baud and Karin, 2001; Chen and Goeddel, 2002).

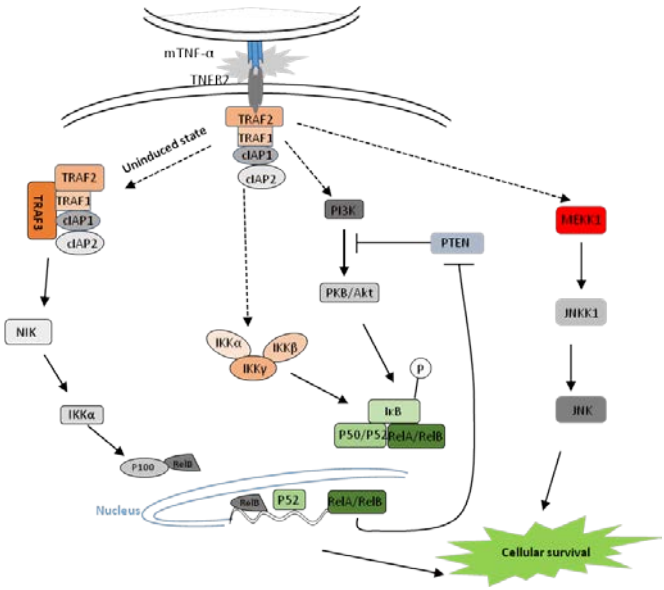

Figure 2.2. Multiple forms of TNFR2 signalling activation are mediated through intracellular protein complex assembly. The transmembrane trimeric TNF-α engagement of TNFR2 leads to cellular survival via the distinct complex signalling pathways. PKB/Akt signalling is described in (Marchetti et al., 2004; Naudé et al., 2011).

TRAF2, TRAF1 and cIAP play a pivotal role not only in TNFR1 signalling pathways but also in TNFR2 pathways. This implicates that there could be a crosstalk between the two receptors (Naudé et al., 2011). It has been demonstrated that the depletion of TRAF2 induced by TNFR2 activation specifically accelerates the TNFR1-dependent caspase 8 activation (Fotin-Mleczek et al., 2002; Li et al., 2002; Wang et al., 1998b). TNFR2 signalling in a certain circumstance may therefore enhance TNFR1-mediated apoptosis by caspase 8 activation.Crosstalk between TNFR1 and TNFR2 is complicated and dependent on the physiologic environment and signalling kinetics between the two receptors. We have previously described TNFR crosstalk in (Naudé et al., 2011).

2.3 TNF AND ITS RECEPTORS—INVOLVEMENT IN NEURODEGENERATIVE DISORDERS

2.3.1 Alzheimer's Disease

AD is a progressive neurodegenerative disorder, and the most common cause of dementia (Qiu et al., 2009). Besides the well-known pathological hallmarks of AD-including the formation of toxic aggregates of amyloid beta (Aβ) and hyperphosphorylated tau proteins-neuroinflammation was more recently described to play a fundamental role in the pathophysiological processes of AD, in which TNF-α in particular could be an important mediator (McAlpine and Tansey, 2008). Evidence for the involvement of TNF-α in AD emanates from various research disciplines. On a genetic level, multiple polymorphisms in the TNF-α gene may be associated with the risk to develop AD (Di Bona et al., 2009). For example, the TNF-α G308A promoter polymorphism, which may cause higher TNF-α expression levels, has been found to increase the risk of AD in certain populations (Kang et al., 2014; Lee et al., 2015; Wang, 2015). At the protein level, plasma and serum TNF-α protein levels are elevated in AD (Alvarez et al., 2007; Bruunsgaard et al., 1999; Fillit et al., 1991). Moreover, TNF-α levels in AD brain tissue were found to be increased, originating from microglia surrounding Aβ plaques (Benzing et al., 1999; McGeer and McGeer, 2003; Zhao et al., 2003). Besides promoting ongoing pro-inflammatory processes in the AD brain, increased TNF-α levels can also affect the accumulation of Aβ. For example, it has been suggested that higher levels of pro-inflammatory cytokines may interfere with phagocytosis of fibrillar Aβ via mechanisms that need further clarification (Koenigsknecht-Talboo and Landreth, 2005). Yet, in monocyte derived macrophages it was shown that cytokines, including TNF-α, could directly decrease the expression of mediators involved in degradation of aggregated proteins, like insulin degrading enzyme, thereby interfering with breakdown of fibrillary Aβ (Yamamoto et al., 2008). Moreover, TNF-α may increase Aβ production by enhancing beta-secretase (BACE1) expression (via NF-κB dependent pathways) and activity, and stimulating gamma-secretase activity via JNK-dependent MAPK signalling (He et al., 2007; Liao et al., 2004).

The extents of signalling through TNFR1 and TNFR2 are an important aspect to consider when interpreting the role of increased TNF-α in AD. TNFR1 protein levels in human post-mortem AD brain tissue are significantly increased as compared to non-demented age-matched controls, while TNFR2 protein levels are decreased (Cheng et al., 2010a; Zhao et al., 2003). Moreover, it appeared that TNF-α in the AD brain has an increased binding affinity to TNFR1, but a decreased affinity for TNFR2 (Cheng et al., 2010b). Interestingly, Zhao et al. (2003) also reported significantly increased levels of TRADD and caspase-3 in AD brains. Data from these studies suggest a shift towards TNFR1-mediated signalling in AD. On a genetic level, a polymorphism in exon 6 of the TNFR2 gene is associated with late-onset AD (Perry et al., 2001). The functional consequences of this polymorphism in TNFR2 however remain, to our knowledge, unclear.

Studies using AD mouse models also have aimed to further the understanding of the role of TNF-α receptors in AD pathology. Montgomery et al. found that deletion of both TNFR1 and TNFR2 in triple-transgenic AD mice (3xTg-AD) significantly exacerbated AD pathology (Montgomery et al., 2011). This suggests that total blockage of TNF-α signalling is not beneficial in this condition, and that both TNFRs should be appreciated separately. Interestingly, Montgomery et al. supported this idea, by showing that silencing of TNFR2 aggravates TNFR1-mediated Aβ and tau pathology in aged 3xTg-AD mice (Montgomery et al., 2013). Moreover, knock down of either TNFR2 or both TNF-α receptors caused enhanced neuroinflammation. Likewise, the group of Shen recently reported that genetic deletion of TNFR2 enhances AD pathology in the APP23 mouse model for AD, while TNFR2 overexpression can reverse these findings (Jiang et al., 2014). The same group also showed that genetic deletion of TNFR1 resulted in inhibition of Aβ production in APP23 mice, and prevented learning and memory deficits (He et al., 2007). McAlpine et al. (2009a) showed that inactivation of TNFR1 signalling diminished Aβ pathology in 3xTg-AD mice, and that administration of inhibitors of sTNF-α (which predominantly activates TNFR1) had similar beneficial effects in 3xTg-AD mice. Moreover, intracerebroventricular injection of oligomeric Aβ resulted in cognitive decline in wildtype mice, but did not affect cognition in TNFR1 knockout mice (Lourenco et al., 2013). Finally, in an *in vitro* study with the SH-SY5Y neuroblastoma cell line, silencing of TNFR2 aggravated the neurotoxic effect of Aβ (Shen et al., 1997). These findings overall seem to support the hypothesis that increased TNFR1 signalling and/or decreased TNFR2 signalling may play an important role in AD pathology.

2.3.2 Parkinson's Disease

Neuroinflammation-besides the aggregation of alpha-synuclein (α-synuclein) proteins – also plays an important role in PD, by directly or indirectly contributing to the degeneration and death of dopaminergic neurons in the substantia nigra. The role of TNF-α and its receptors in PD were also previously reviewed by McCoy and Tansey (McCoy and Tansey, 2008). TNF-α levels are significantly increased in the brain and CSF of PD patients, and increased TNFR1 levels were found in the substantia nigra of PD patients (Mogi et al., 2000). Evidence for the involvement of TNF-α and its receptors in mechanisms of PD progression is described in studies with different PD animal models and *in vitro* models. For example, some models aim to mimic the α-synucleinopathy that is observed in PD, by

overexpressing wildtype or mutant α-synuclein. *In vitro*, BV2 cells (a murine microglial cell line) showed elevated TNF-α secretion upon α-synuclein overexpression (Rojanathammanee et al., 2011), and primary murine microglia presented a significant increase in TNF-α expression after exposure to mutant α-synuclein (Su et al., 2009). *In vivo*, overexpression of α-synuclein via recombinant adeno-associated virus (AAV-synuclein) injection into the substantia nigra was also found to increase TNF-α expression (Chung et al., 2009; Theodore et al., 2008). Another PD model makes use of 6-hydroxydopamine (6-OHDA); a toxic dopamine analogue which leads to dopaminergic neuron death upon administration. *In vitro*, it was shown that selective activation of TNFR2 (by TNC-scTNFR2, a TNFR2-specific agonist) rescued cultured neurons from 6-OHDA-induced cell death (Fischer et al., 2011). *In vivo*, peripheral and intranigral injection in rats with specific inhibitors of sTNF-α (XPro-1595 and XENP345, respectively) showed to reduce 6-OHDA-induced death of dopamine neurons (Barnum et al., 2014a; Harms et al., 2011; McCoy et al., 2006, 2008). Considering that sTNF-α preferably binds and activates TNFR1 rather than TNFR2, and that TNFR1 is highly expressed by dopamine neurons, it was suggested that the neuroprotective effects of these sTNF-α blockers may have resulted mostly from attenuated signalling via TNFR1 (McCoy et al., 2006; Probert, 2015). These findings indicate that TNF-α and its receptors exert similar functions in PD as previously described for AD; TNF-α functioning is shifted towards increased TNFR1 signalling and certain neuroprotective effects induced via TNFR2 signalling are decreased. It should be noted however that studies using mice with TNFR1 and/or TNFR2 deletions have led to contradictory findings about their roles in PD pathology, which may be due to differences in the PD models used, as reviewed in (McCoy and Tansey, 2008). For example, it was shown that mice lacking both TNFR1 and TNFR2 in the 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP, a dopaminergic neurotoxin) model for PD were completely protected against the dopaminergic neurotoxicity of MPTP, while mice lacking either TNFR1 or TNFR2 were not protected (Sriram et al., 2002).

2.3.3 Ischemic Stroke

Ischemic stroke can arise when a blood vessel supplying blood to the brain is obstructed. Sudden loss of blood flow to a brain region causes damage and cell death in the (nutrients and oxygen-) deprived area. Upon ischemic stroke, different brain cell types in proximity of the ischemic lesion site (including neurons, microglia and astrocytes) increase their production of TNF-α. This has been shown in human brain tissue as well as in experimental animal models of stroke (Dziewulska and Mossakowski, 2003; Liu et al., 1994; Sairanen et al., 2001; Tuttolomondo et al., 2008). As reviewed by Pan and Kastin, TNF-α was shown to have both detrimental and beneficial effects in stroke (Pan and Kastin, 2007). Several studies reported that inhibition of TNF-α (e.g., by etanercept, a human TNFR2-IgG Fc fusion protein) reduces infarct size and neuroinflammation (Arango-Dávila et al., 2014; Sumbria et al., 2012; Tobinick et al., 2012; Wu et al., 2015), while on the other hand complete knockout of both TNFRs increases the sensitivity for stroke and aggravates neuronal damage (Bruce et al., 1996). Moreover, in stroke *in vitro* and animal models, pretreatment with TNF-α (which models ischemic preconditioning), mediates

neuroprotective effects after ischemia (Ding et al., 2006; Nawashiro et al., 1997). In accordance with the above-discussed disorders, complete abolition of TNF-α signalling, as well as exaggerated TNF-α signalling are detrimental in ischemic stroke. This may likely depend on the contribution of TNF-α/TNFR1 and TNF-α/TNFR2 signalling. In addition to TNF-α, its receptors are also up-regulated in stroke. These findings imply a neuroprotective role for TNFR1, while TNFR2 signalling may have detrimental effects by aggravating neuroinflammatory processes. However, conflicting data is also present. For example, our group previously showed that absence of TNFR1 in mice strongly reduced neurodegeneration after retinal ischemia-reperfusion, while lack of TNFR2 exacerbated neurodegeneration (Fontaine et al., 2002). This finding indicates that TNFR1 signalling may augment neuronal death and TNFR2 may promote neuroprotection. In accordance, an *in vitro* study with the SH-SY5Y neuroblastoma cell line showed that silencing of TNFR2 aggravated cell injury upon hypoxic conditions (Shen et al., 1997). Moreover, in the immature brain, TNFR1-JNK signalling was responsible for neuroinflammation and neurovascular damage in lipopolysaccharide (LPS)-sensitized hypoxic-ischemia brain injury (Wang et al., 2014). The different models of ischemia that were used may in part explain these contradictory findings. Furthermore, the duration of the induced ischemia (acute/transient *vs*. chronic) might potentially affect the pathways, kinetics and outcomes of TNF-signalling. Also, the acuteness of ischemic lesions may explain differences between ischemic stroke and other neurodegenerative disorders like AD and PD, which gradually develop over a longer period of time. In disorders that develop slowly, the expression levels and distribution of TNFRs may gradually change during the pathological process (e.g. resulting in lower TNFR2 expression in AD brains). This might explain how acute insults could result in different effects of TNF-α, as compared to conditions in which lesions arise over a longer period of time.

Table 2.1. Summary of the TNF-α family members that are subject of this review, and their roles in neurodegenerative conditions.

TNF-α member	Tissue	Finding	Model	Ref.
TNF-α	CNS	TNF-α protein levels are increased in AD brain tissue.	Human AD patients.	(Benzing et al., 1999; McGeer and McGeer, 2003; Zhao et al., 2003)
	Plasma, serum	TNF-α protein levels are increased in AD plasma and serum.	Human AD patients.	(Alvarez et al., 2007; Bruunsgaard et al., 1999; Fillit et al., 1991)

TNFR1 and TNFR2	CNS	TNFR1 protein levels are increased, TNFR2 protein levels are decreased.	Human AD patients.	(Cheng et al., 2010a; Zhao et al., 2003)
		Deletion of both TNFRs exacerbates AD pathology.	3xTg-AD mouse model.	(Montgomery et al., 2011)
		Silencing or deletion of TNFR2 aggravates AD pathology. TNFR2 overexpression reverses these effects.	3xTg-AD mouse model and APP23 mouse model.	(Montgomery et al., 2013)(Jiang et al., 2014)
		In vitro, TNFR2 silencing promotes Aβ neurotoxic effects.	SH-SY5Y cell line.	(Shen et al., 1997)
		Deletion of TNFR1 diminishes AD pathology.	3xTg-AD mouse model and APP23 mouse model.	(He et al., 2007)(McAlpine et al., 2009a).
		sTNF-α inhibitors diminish AD pathology.	3xTg-AD mouse model.	(McAlpine et al., 2009a)
sTNFR1 and sTNFR2	CSF, serum and plasma	sTNFR1 levels are increased. sTNFR2 levels are unchanged or decreased.	Human control and MCI patients.	(Bai et al., 2013; Diniz et al., 2010; Faria et al., 2014; Jiang et al., 2011; Sun et al., 2014)
		Higher sTNFR1 serum levels can predict conversion from MCI to AD.	Human control and MCI patients.	(Diniz et al., 2010)

		sTNFR1 and sTNFR2 levels correlate with BACE1 activity and Aβ40 levels, as well as with tau CSF levels.	Human control and MCI patients.	(Buchhave et al., 2010)
TNF-α	CNS and CSF	TNF-α levels are increased in brain and CSF.	Human control and PD patients.	(Rojanathammanee et al., 2011;Chung et al., 2009; Theodore et al., 2008)
TNFR1	CNS	TNFR1 levels are increased in the substantia nigra.	Human control and PD patients.	(Mogi et al., 2000)
		sTNF-α inhibitors reduce cell death of dopamine neurons.	Rat 6-OHDA toxicity model.	(Barnum et al., 2014a; Harms et al., 2011; McCoy et al., 2006, 2008)
TNFR2	CNS	Selective activation of TNFR2 protects dopaminergic neurons.	Neuronal culture, 6-OHDA toxicity model.	(Fischer et al., 2011)
TNFR1 and TNFR2	CNS	Deletion of both TNFRs protects from dopaminergic toxicity, while lack of either TNFRs alone is not protective.	Mouse, MPTP toxicity model.	(Sriram et al., 2002)
sTNFR1	Serum and plasma	Serum sTNFR1 levels are increased.	Human control and PD patients.	(Hirsch and Hunot, 2009; Mogi et al., 1994; Scalzo et al., 2009)
		Higher serum sTNFR1 correlate with a later	Human control and PD patients.	(Scalzo et al., 2009)

		onset of sporadic PD.		
		Elevated plasma sTNFR1 levels predict poorer executive functioning in PD.	Human control and PD patients.	(Rocha et al., 2014)
TNF-α	CNS	TNF-α production is increased around the lesion site.	Human brain tissue and animal models of stroke.	(Dziewulska and Mossakowski, 2003; Sairanen et al., 2001; Tuttolomondo et al., 2008)
		Inhibition of TNF-α reduces infarct size and neuroinflammation.	Stroke mouse models.	(Arango-Dávila et al., 2014; Sumbria et al., 2012; Tobinick et al., 2012)
TNFR1	CNS	TNFR1 knockout mice have larger infarct sizes compared to wildtype and TNFR2 knockout mice.	Stroke mouse model.	(Gary et al., 1998;Lambertsen et al., 2009)
		TNFR1 is responsible for expression of neuroprotective factors upon ischemia.	Stroke mouse model.	(Taoufik et al., 2007, 2008)
		Absence of TNFR1 reduces retinal Ischemia-reperfusion damage.	Mouse retinal ischemia-reperfusion model.	(Fontaine et al., 2002)
		TNFR1 signalling causes neuroinflammation and neurovascular damage in	LPS-sensitized hypoxic-ischemia mouse model.	(Wang et al., 2014)

		the immature brain.		
TNFR2	CNS	Absence of TNFR2 aggravates retinal ischemia-reperfusion damage.	Mouse retinal ischemia-reperfusion model.	(Fontaine et al., 2002)
		TNFR2 silencing increases cell injury upon hypoxic conditions.	SH-SY5Y cell line.	(Shen et al., 1997)
		TNFR2 signalling can result in inflammatory ischemia.	Stroke mouse model.	(Akassoglou et al., 2003)
TNFR1 and TNFR2	CNS	Deletion of both TNFRs aggravates neuronal damage.	Stroke mouse model.	(Bruce et al., 1996)
TNF-α	CNS	TNF-α levels are increased in MS lesions.	Human MS brain tissue.	(Hofman et al., 1989; Selmaj et al., 1991)
		Constitutive TNF-α overexpression can cause a spontaneous inflammatory demyelinating disorder.	TNF-overexpressing mouse model.	(Probert et al., 1995)
		TNF-α knockout increases demyelination and inflammation.	EAE mouse model.	(Constantinescu et al., 2011; Liu et al., 1998)
		TNF-α knockout delays both demyelination and	Cuprizone mouse model.	(Arnett et al., 2001)

		remyelination.		
TNFR1	CNS	TNFR1 knockout mice do not develop EAE or have a less severe disease course.	EAE mouse model.	(Eugster et al., 1999; Kassiotis and Kollias, 2001; Suvannavejh et al., 2000; Williams et al., 2014)
		TNFR1 signalling induces oligodendrocyte apoptosis and primary demyelination.	TNF-transgenic mice.	(Akassoglou et al., 1998)
		TNFR1 may contribute to inflammatory infiltration of the spinal cord.	EAE mouse model.	(Gimenez et al., 2006)
		Selective inhibition of TNFR1 signalling ameliorates EAE-induced pathology.	EAE mouse model.	(Nomura et al., 2011; Williams et al., 2014)
		sTNF-α inhibition protects against EAE symptoms.	EAE mouse model.	(Brambilla et al., 2011; Taoufik et al., 2011)
TNFR2	CNS	TNFR2 knockout mice show aggravated demyelination and disease symptoms.	EAE mouse model.	(Eugster et al., 1999; Kassiotis and Kollias, 2001; Suvannavejh et al., 2000; Williams et al., 2014)
		TNFR2 signalling mediates remyelination and oligodendrocyte precursor cell proliferation.	Cuprizone mouse model.	(Arnett et al., 2001)

		Selective stimulation of TNFR2 protects primary oligodendrocytes from oxidative stress.	Primary oligodendrocyte cell culture.	(Maier et al., 2013)

2.3.4 Multiple Sclerosis

MS is a chronic demyelinating disease of the CNS, resulting in disrupted nerve signalling and thereby a wide range of neurological symptoms. It has been suggested that the demyelination of axons is due to the death of myelin-forming oligodendrocytes, which in part may be caused by detrimental inflammatory and immune responses targeted to these cells (Cudrici et al., 2006). The involvement of TNF-α in MS has been explored in several studies (McCoy and Tansey, 2008). Increased TNF-α levels were found in MS lesions (Hofman et al., 1989; Selmaj et al., 1991). In a transgenic mouse model that overexpresses murine TNF-α specifically in the CNS, it was demonstrated that constitutive TNF-α expression leads to spontaneous development of a chronic inflammatory demyelinating disorder (Probert et al., 1995). In addition, peripherally increased TNF-α levels have been associated with synaptic instability in the brain, and as such may contribute to sensory and cognitive impairments as seen in MS (Yang et al., 2013). On the other hand, complete knockout of TNF-α in experimental autoimmune encephalomyelitis (EAE, an MS animal model) mice caused deleterious effects, including increased inflammation, demyelination and higher mortality as compared to control mice (Constantinescu et al., 2011; Liu et al., 1998). In the cuprizone model (a toxin causing reversible demyelination), complete knockout of TNF-α in mice resulted in delayed demyelination (suggesting that TNF-α promotes acute demyelination) as well as delayed remyelination (suggesting that in later stages, TNF-α promotes remyelination) (Arnett et al., 2001). The beneficial versus detrimental effects of TNF-α in MS may greatly depend on its signalling via either TNFR1 or TNFR2. Akassoglou et al. (1998) demonstrated a dominant role for TNFR1 signalling in TNF-mediated oligodendrocyte apoptosis and primary demyelination. In addition, TNFR1 was suggested to contribute to inflammatory infiltration of the EAE spinal cord (Gimenez et al., 2006). Interestingly, it was recently shown in different studies that administration of an antagonistic antibody that selectively targets TNFR1 ameliorated disease symptoms in the EAE mouse model (Nomura et al., 2011; Williams et al., 2014). Also, inhibition of sTNF-α by XPro-1595 protected EAE mice from clinical symptoms and improved axon preservation and remyelination, indicating a detrimental effect of sTNF-α (which signals mostly via TNFR1) (Brambilla et al., 2011; Taoufik et al., 2011). Furthermore, studies showed that TNFR1 knockout mice do not

develop EAE, or have a less severe disease course. TNFR2 knockout mice on the other hand were seen to develop more extensive demyelination and aggravated EAE disease symptoms (Eugster et al., 1999; Kassiotis and Kollias, 2001; Suvannavejh et al., 2000; Williams et al., 2014). Similarly, in the cuprizone model, TNFR2 showed to be responsible for TNF-α mediated remyelination and proliferation of oligodendrocyte precursor cells (Arnett et al., 2001). A neuroprotective role of TNFR2 on oligodendrocyte progenitor cells was also directly shown in *in vitro* studies by Maier et al. (2013). In this study, primary oligodendrocytes from transgenic mice expressing human TNFR2 were shown to be protected from oxidative stress, after preconditioning the cells with a TNFR2 specific agonist. This protective effect might be elicited by TNFR2-mediated induction of anti-apoptotic and cell survival genes (Maier et al., 2013). Taking together the above results, it may not be a surprise that general blockage of TNF-α signalling can have a net detrimental effect, by also inhibiting the neuroprotective signalling of TNF-α. This idea is supported by different studies, including reports in which administration of TNF-α antagonists was associated with the onset of MS (Pfueller et al., 2008; Sicotte and Voskuhl, 2001). In general, specifically blocking TNFR1 or stimulating TNFR2 signalling may provide a promising therapeutic possibility in MS. It should be noted however that TNFR1 might also have beneficial effects in MS. For example, it has been suggested that TNFR1 signalling is important for the onset of EAE, but also for limiting EAE progression at a later stage (Probert, 2015). Therefore, specific modulation of TNF-α receptor mediated signalling at specific stages of the disease may be a promising approach for effective outcomes in MS.

2.3.5 Other Neurodegenerative Disorders

Besides the four conditions described above, there are certainly many other disorders with neurodegenerative features in which a role for TNF-α has become clear. Although for this review it was chosen to focus on AD, PD, ischemic stroke and MS, investigation of TNF-α and its receptors in other neurodegenerative conditions may also greatly contribute to the understanding of TNF-α's effects, and the factors on which these effects depend. Examples of other disorders in which TNF-α has been implicated include traumatic brain injury (TBI) (Woodcock and Morganti-Kossmann, 2013), epilepsy (Li et al., 2011) and Huntington's disease (HD) (Alto et al., 2014; Ellrichmann et al., 2013). Evidence exists that TNFR1 signalling may exacerbate cognitive dysfunction in a mouse model of TBI, while TNFR2 signalling may attenuate it (Longhi et al., 2008, 2013). Also in models of epileptic seizures, inhibition of TNFR1 signalling as well as activation of TNFR2 were suggested to protect against seizure-induced neuronal damage (Balosso et al., 2005; Li et al., 2011; Thompson et al., 2011). In *in vitro* and *in vivo* models of HD, blockage of sTNF-α by XPro-1595 showed to reduce different pathological features of HD (Hsiao et al., 2014). Another group of diseases in which TNF-α signalling may play an important role is lysosomal storage disease (LSD). LSDs are rare, and result from mutations in lysosomal enzyme-encoding genes, causing the enzyme's substrate to accumulate in the lysosomes. Depending on the enzyme that is affected, different substrates may pile up, leading to different LSDs. More than thirty LSDs are known, and include for example Fabry disease, Pompe disease, Gaucher disease and Niemann-Pick type C (NP-C) disease (Neufeld, 1991).

In the majority of LSDs neurodegeneration occurs, with neuroinflammatory processes being implicated as important contributors herein (German et al., 2002; Patel et al., 1999; Platt et al., 2012). In human NP-C patient brain tissue as well as in a mouse model of this disease, apoptotic neurons were detected. Interestingly, in affected brain regions in an NP-C mouse model, the expression of different components in TNF-mediated apoptotic signalling was found to be increased, including that of TNF-α itself, TNFR1 and caspase-8 (Wu et al., 2005). Gaucher disease patients showed elevated serum TNF-α levels, and in the fetal brains of a Gaucher disease mouse model, TNF-α levels (as well as the levels of different other pro-inflammatory cytokines) were increased (Barak et al., 1999; Hong et al., 2006). Also after birth, TNF-α and TNFR1 were found to be up-regulated in the brains of Gaucher disease mice with increasing age and disease severity (Vitner et al., 2012). All in all, there are many conditions with neurodegenerative components in which TNF-α signalling may significantly contribute to neuropathological processes, and more research focusing on TNF-α signalling via either TNFR1 or TNFR2 is warranted.

2.4 TNFR1 AND TNFR2 MEDIATED SIGNALLING IN NEURODEGENERATION

Despite the overlap between the signalling pathways of TNFR1 and TNFR2, their effects can differ greatly. For example, although both receptors may cause activation of transcription factor NF-κB (however with different activation kinetics (Marchetti et al., 2004), different genes are transcribed, depending on which TNFR was activated. The involvement of possible TNFR1 and TNFR2 downstream signalling pathways in neurodegenerative disorders will be discussed based on studies using TNFR1- and/or TNFR2-specific knockout animals, or compounds targeting TNFRs specifically.

2.4.1 TNFR1 - Possible Downstream Targets in Neurodegeneration

Neutrophil gelatinase-associated lipocalin (NGAL, also known as lipocalin 2; the murine orthologue of NGAL) is a recently described downstream product of TNFR1-mediated signalling (Naudé et al., 2012). NGAL plays a role in the innate immune system, and is important in the defence against certain bacteria (Goetz et al., 2002). Mounting evidence recently provided insights into interesting functions of NGAL in the brain, particularly its role in neurodegenerative disorders. Robust increased NGAL protein levels were found in AD post mortem human brain tissue (Naudé et al., 2012), a mouse model for MS (Marques et al., 2012) and mouse model for cerebral ischemia (Wang et al., 2015). Increased NGAL protein levels are detrimental to neuronal health (Bi et al., 2013) and sensitize neurons and other brain cell types to cell death upon exposure to Aβ and oxidative stress (Lee et al., 2012; Mesquita et al., 2014; Naudé et al., 2012). In addition, NGAL was shown to further promote pro-inflammatory reactions, and to stimulate classical inflammatory activation of microglia and astrocytes (Jang et al., 2013a, 2013b). NGAL plays an interesting role in TNF-α mediated signalling pathways. Our research group showed that NGAL is solely increased and secreted upon TNFR1 stimulation in murine primary neurons, astrocytes and microglia

cells. Increased NGAL in turn silences the TNFR2-mediated PI3K-PBK/Akt pathway in neurons, possibly by increasing PTEN levels (Naudé et al., 2012). Thus, NGAL may play an important role in shifting TNF-α signalling towards TNFR1-mediated pathways observed in different neurodegenerative conditions, as previously described in this review.

Different studies demonstrated that TNFR1 can induce matrix metalloproteinase 9 (MMP-9) expression (Lee et al., 2010; Lin et al., 2008; Tsai et al., 2014). In the A549 cell line (a human lung adenocarcinoma epithelial cell line) TNF-α can induce MMP-9 expression via a TNFR1/TRAF2/PKC alpha dependent pathway (Lee et al., 2010). MMP-9 has been associated with different physiological and pathophysiological processes. For example, MMP-9 was shown to be able to interact with Aβ, and can play a role in disruption of the blood-brain barrier (Lakhan et al., 2013; Mizoguchi et al., 2009; Takata et al., 2011; Yan et al., 2006). This latter effect seems to be associated with MMP-9's actions in degradation of the extracellular matrix. Of note, it was shown that MMP-9 can form a complex with NGAL, and that NGAL may elongate the activity of MMP-9 (Yan et al., 2001).

TNR1 could engage in processes concerning the clustering of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (AMPAR). Deletion of TNFR1 was found to suppress excitatory synaptic transmission via the localization of AMPA receptors to the synapses of cortical neurons (He et al., 2012). It was therefore suggested that TNFR1 might be involved in AMPAR-mediated excitotoxicity. Furthermore, it was shown that TNFR1 signalling induces excitotoxicity by promoting glutamate release in mouse primary microglia and astrocyte cell culture (Bezzi et al., 2001; Olmos and Lladó, 2014; Takeuchi et al., 2006). More specific to AD, TNFR1 was found to be involved in processing of the amyloid precursor protein (APP) and Aβ plaque formation, by increasing BACE1 promotor activity (He et al., 2007).

2.4.2 TNFR2 – Possible Downstream Targets in Neurodegeneration

Examples of TNFR2-specific signalling in neurodegeneration have also been described (Fontaine et al., 2002; Marchetti et al., 2004). Dolga et al. identified certain small conductance calcium-activated potassium K(Ca)2 channels as downstream products of TNFR2 signalling (Dolga et al., 2008a, 2011). These channels contribute to neuroprotection against neuronal overstimulation, by lowering the neuronal excitability. It is specifically the expression of K(Ca)2.2 that was shown to be downstream of TNF-α/TNFR2 signalling, via NF-κB activation. As such, it was suggested that in primary cortical neurons, TNF-α induces K(Ca)2.2 channel activation, resulting in neurons that are more resistant to excitotoxic cell death by preventing intracellular calcium levels to become pathologically high (Dolga et al., 2008a, 2011). In the past years it has been shown that K(Ca)2 channels may also be involved in mechanisms contributing in plasticity of the hippocampal CA1 neurons and in learning and memory (Allen et al., 2011; Kuiper et al., 2012).

Fischer et al. (2014) showed that TNFR2-mediated activation of the PI3K-PKB/Akt pathway in primary astrocytes induces the expression of different neuroprotective genes, including the gene that encodes leukemia inhibitory factor (LIF). LIF is a neurotrophic cytokine,

which in the brain is mainly produced by astrocytes. Elevated levels of LIF were shown to promote the maturation of oligodendrocytes (Fischer et al., 2014), and LIF was found to protect primary neurons against excitotoxicity (Moidunny et al., 2012). Moreover, in the EAE mouse model LIF was demonstrated to protect axons in the brain from acute inflammatory damage (Gresle et al., 2012).

Other downstream products of TNFR2 signalling include CXCL12, which has also been implicated in the proliferation and differentiation of oligodendrocyte progenitor cells (Patel et al., 2012). Moreover, its levels were found down regulated in the Tg2576 AD mouse model, and reduced CXCL12 levels were shown to cause impairments in learning and memory (Parachikova and Cotman, 2007). Lastly, TNFR2 signalling in microglia has been related with induction of anti-inflammatory pathways, for example the upregulation of IL-10 (Veroni et al., 2010).

All examples discussed in this chapter support the idea that TNFR1 may specifically induce factors that contribute to neurodegenerative processes, while TNFR2 specifically mediates factors involved in neuroprotective mechanisms (Figure 2.3). However, in agreement with the rest of this review, things are certainly far more complex. Therefore, different unknowns and contradictory findings underlining and adding to the complexity of TNF-signalling will be addressed in the following chapter.

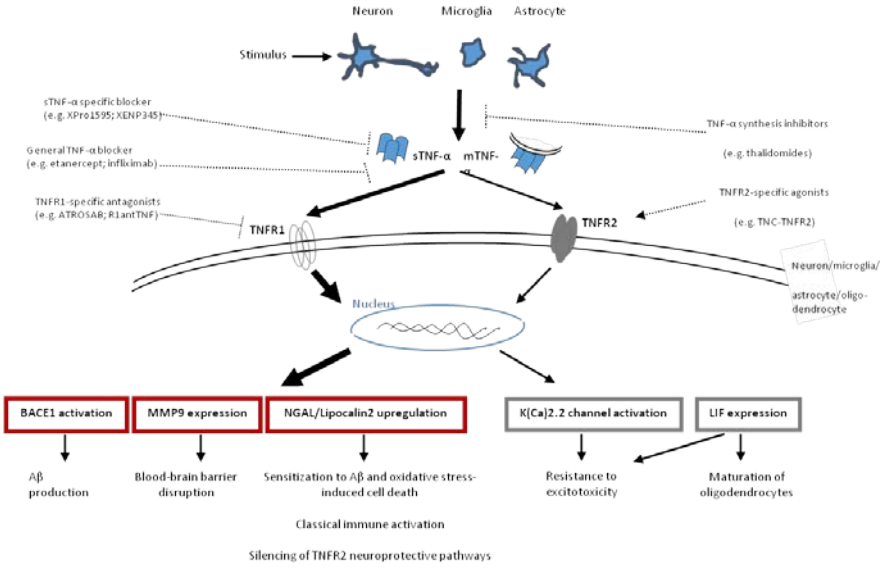

Figure 2.3. Examples of specific downstream targets of TNFR1 and TNFR2 that may be involved in neurodegenerative disorders. Dotted lines indicate existing potential therapeutic approaches. Other general TNF-α blockers, not depicted in this figure, include adalimumab, certolizumab pegol and golimumab. Other new TNFR1-specific antagonists include DMS5540 and TROS (also see review by Fischer et al.(2015)).

2.5 COMPLEX MATTERS: TNFR1 SIGNALIING IS PRIMARILY DAMAGING AND TNFR2 BENEFICIAL?

Although in this paper we generally highlighted an important neuroprotective role for TNFR2 and a neurodegenerative role for TNFR1 in different neurodegenerative disorders, findings that contradict this idea cannot be ignored. As described in the previous paragraphs, for several neurodegenerative disorders conflicting results have been found concerning beneficial/detrimental effects of TNFR1 and TNFR2 signalling. These contradictory findings emphasize the complexity of TNFR1/TNFR2 signalling, and confirm that our understanding of TNF-signalling is still work in progress.

The outcome of TNF-α signalling will likely depend on many variables: the availability of both receptors, the available levels of sTNF-α and mTNF-α ligands, the availability of the components in their respective signalling cascades, and the level of crosstalk between the two pathways, all of which likely depend on the cell type, tissue and the condition. Indeed, different studies have suggested that TNF-α signalling may be strongly brain region-specific, which may in part depend on, e.g., the relative density and activity of microglia in specific regions (Rodriguez et al., 2009; Sriram et al., 2006; Veroni et al., 2010). Also the timing of TNF-α signalling in different stages of neurodegeneration could affect the outcomes of TNF-α. For example, TNF-α mediates a neuroprotective effect when hippocampal organotypic slice cultures are treated with TNF-α before an ischemic insult (which may simulate ischemic preconditioning), but has neurotoxic effects when administered after the same ischemic insult (Wilde et al., 2000). Furthermore, TNFR2-mediated pathways have receivedless attention compared to TNFR1 and undefined signalling mechanisms possibly remain to be discovered. These and other factors greatly increase the complexity of TNF-signalling, and a few will be further discussed below.

2.5.1 Selective Harmful Downstream Targets of TNFR1 and Beneficial Downstream Targets of TNFR2?

Although we described examples supporting the presence of potent detrimental downstream signalling pathways mediated specifically via TNFR1, while TNFR2 signalling may mostly activate genes with potential neuroprotective functions, matters are often not that clear-cut. For instance, it should be noted that there are certain examples available of neuroprotective pathways induced via TNFR1, and damaging outcomes of TNFR2 signalling. For example, a known downstream target of TNFR1 is nerve growth factor (NGF), which is important for survival and growth of neurons (Kuno et al., 2006), while TNFR2 may also promote expression of potentially detrimental factors, such as intercellular adhesion molecule-1 (ICAM-1), which has been associated with neuroinflammation and neurodegeneration (Lucas et al., 1997; Pola et al., 2003). Moreover, the specificity of certain downstream products to either TNFR1 or TNFR2 is sometimes unclear. For example, some studies have implied NGF to be solely induced via TNFR1 in fibroblasts, while it appeared in astrocytes that signalling via both receptors can cause NGF production (Hattori et al., 1996; Kuno et al., 2006). Such contradictory findings may

amongst others depend on variables like the cell type studied, the proximate cellular conditions, TNFR1-TNFR2 signalling kinetics and crosstalk between the receptors. In addition, although the above described processes were found to be specifically induced via either TNFR1 or TNFR2, there may very well be multiple other (TNF-α-independent) pathways that may influence TNF-α mediated pathways and their outcomes. For example, it may be good to keep in mind that lymphotoxin α (TNF-β) can also bind both TNFR1 and TNFR2. Comparable to TNF-α, lymphotoxin α also has been implicated in different processes in the brain, and in the pathogenesis of conditions including MS (Dopp et al., 1997; Etemadi et al., 2013).

2.5.2 Soluble TNF Receptors

Adding to the complexity of TNF-α signalling, besides membrane TNF receptors (mTNFRs), soluble TNF receptors (sTNFRs) also exist, which influence TNF-α signalling mechanisms as well. sTNFRs (sTNFR1 and sTNFR2) can be formed via a process known as ectodomain shedding, in which the extracellular domains of membrane TNFRs are cleaved off by TACE (which thus shows to have more targets besides mTNF-α) and released into the extracellular space. Notably, in addition to ectodomain shedding, it has been described that TNFR1 can also end up in the extracellular space via exocytosis. This generates exome-like vesicles with full-length TNFR1 incorporated in the vesicle membrane (Hawari et al., 2004). The exact functions and mechanisms of actions of sTNFRs (as well as the full-length TNFR1 within vesicles) however remain elusive. Shedding of mTNFRs is thought to regulate the actions of TNF-α, firstly by diminishing the number of mTNFRs on the cell membrane, and secondly by binding of sTNFRs to TNF-α, thereby preventing TNF-α to bind to and activate mTNFRs. Alternatively, it was suggested that sTNFRs bind to sTNF-α and subsequently stabilize and preserve the bio-active trimeric forms of TNF-α (Diez-Ruiz et al., 1995). As such, sTNFRs levels might reflect the activity of TNF-α. Interestingly, intravenous injection of TNF-α in human volunteers suggested that TNF-α is a potent mediator of increased sTNFR1 and sTNFR2 release (Jansen et al., 1995).

Different studies indicate that the levels of sTNFR1 as well as TACE activity are increased in plasma, serum and CSF from AD patients (Bai et al., 2013; Diniz et al., 2010; Faria et al., 2014; Jiang et al., 2011; Sun et al., 2014). Moreover, Diniz et al. (2010) showed that higher serum sTNFR1 levels can predict conversion from mild cognitive impairment (MCI) to AD. Plasma and CSF sTNFR2 levels were found to be increased in AD by some groups (Bai et al., 2013; Jiang et al., 2011), while other studies showed no differences in sTNFR2 levels (Diniz et al., 2010; Faria et al., 2014). Similar to AD, increased serum sTNFR1 levels were found in patients with PD (Hirsch and Hunot, 2009; Mogi et al., 1994; Scalzo et al., 2009). Also, a single nucleotide polymorphism in the gene encoding TNFR1, resulting in production of a soluble form of TNFR1 that is able to antagonize TNF-α, was found to be associated with MS (Gregory et al., 2012). Although in these cases the exact meaning of these increased serum sTNFR1 levels is not clear, it was found that higher serum sTNFR1 levels are associated with a later onset of sporadic PD (Scalzo et al., 2009). In addition to the potential functions and effects of sTNFRs described above, sTNFRs may also influence TNF-α signalling via reverse signalling through mTNF-α.

Besides being a ligand for TNFR1 and TNFR2, mTNF-α can also act as a receptor itself. Thus, in addition to inducing a signalling cascade in TNFR1/TNFR2-expressing cells, mTNF-α can also elicit a signal transduction pathway back into the cell on which it is expressed (Eissner et al., 2004). This process is called reverse signalling, and can occur when mTNF-α is bound by a (s)TNFR or agonistic antibody (Eissner et al., 2004; Sipos et al., 2015). sTNFRs may have significant effects on cells by inducing reverse signalling. For example, it was reported that sTNFR1 can induce apoptosis of monocytes through reverse signalling via mTNF-α. It appeared that this sTNFR1-induced apoptosis is independent of death receptor pathways, but is mediated via autocrine transforming growth factor beta (TGF-β) through p38 MAPK (Waetzig et al., 2005). In addition, it was recently shown in cultured superior cervical ganglion (SCG) neurons that sTNFR1 promotes sympathetic axon growth and branching through reverse signalling via mTNF-α, and relies on downstream activation of ERK (Kisiswa et al., 2013). In addition to endogenous sTNFRs, different TNF-α inhibitors (like etanercept and infliximab) can induce reverse signalling through mTNF-α as well. As suggested in recent studies, effects of such compounds may significantly depend of reverse signalling, besides the effects arising from the prevention of mTNF-α and sTNF-α to bind their TNFRs (Kirchner et al., 2004; Meusch et al., 2009).

Taken together, soluble TNFRs may play a substantial role in how TNF-α interacts and functions with its receptors, possibly especially in a systemic pro-inflammatory environment. This is an interesting and noteworthy research field that can provide important insights in TNF-α functioning. In theory, a potential therapeutic strategy to reduce excessive TNFR1 signalling may be by stimulating TNFR1 ectodomain shedding. Interestingly, some mediators (with aminopeptidase regulator of TNFR1 shedding (ARTS-1) appearing as a key regulator) have been identified that are essential for TNFR1 shedding, but do not affect TNFR2 shedding (Cui et al., 2002; Islam et al., 2006). While ARTS-1 also participates in shedding of other receptors besides TNFR1 (including IL-6R and IL-1R2 (Cui et al., 2003)), downstream effectors of ARTS-1 may prove to be TNFR1-specific, and could provide a therapeutic target.

2.5.3 Interaction between TNFR2 and Interleukin-17 Receptor D

Moreover, the signalling of membrane TNFRs may also hold more complexity than has become clear so far. For example, it was very recently demonstrated that TNFR2 (but not TNFR1) can form a heteromer with interleukin-17 receptor D (IL-17RD, also known as Sef), leading to activation of NF-κB signalling via TRAF2 recruitment (Yang et al., 2015). Depletion of IL-17RD was found to impair TNFR2-mediated activation of NF-κB. The complex between IL-17RD and TNFR2 was shown to be formed in HK-2 and 786-O cell lines (a human proximal tubular cell line derived from normal kidney, and a human renal cell adenocarcinoma cell line, respectively), and also in rat and mouse renal tissue. This indicates that the interaction between TNFR2 and IL-17RD may arise under physiological conditions (Yang et al., 2015).

All these complex issues and unknowns emphasize that gaps remain in the basic understanding of TNF-α signalling, which may challenge the identification of suitable TNF-targeting therapeutics for different neurodegenerative disorders.

2.6 TARGETING TNF ALPHA SIGNALLINGS: AN OPPORTUNITY FOR TREATMENT OF NEAURODEGENERATIVE DISORDERS?

As concluded in this review, targeting TNF-α signalling towards its neuroprotective functioning could provide potent therapeutic strategies for patients with neurodegenerative disorders. It seems that therapeutics could aim at a certain step of TNF-α mediated signalling pathways via inhibiting detrimental pathways.

2.6.1 Targeting TNF-α as Treatment for Neurodegenerative Disorders

Because TNF-α is a key inductor in some inflammatory disease such as rheumatoid arthritis (RA), psoriatic arthritis (PsA), Crohn's disease (CD) as well as some neurodegenerative disorders, directly inhibiting this cytokine could prevent or treat those conditions. A number of researchers have demonstrated that directly blocking the actions of TNF-α via anti-TNF-α antibodies and its antagonists indeed can ameliorate inflammatory and neurodegenerative disorders (Liu et al., 2015; McAlpine et al., 2009b; Mease, 2002). Additionally, inhibition of TNF-α not only prevents the course of MS but also promotes axon preservation and remyelination in EAE mouse model (Brambilla et al., 2011). Due to its therapeutic capability, treatments aimed to inhibit the functions of TNF-α have been shown as effective treatment strategy of RA, PsA as well as CD in experimental trials and are currently being used worldwide in the clinical setting (Locksley et al., 2001; Maini and Taylor, 2000; Spinelli et al., 2014; 2015). So far, pharmaceutical anti-TNF-α agents that include thalidomide analogues which inhibit the production of TNF-α have been applied for the treatment of RA (Greig et al., 2004; Tweedie et al., 2007; Zhu et al., 2003). Moreover, other pharmaceutical agents including etanercept, adalimumab and infliximab, that can bind both sTNF-α and mTNF-α thereby inhibiting their signalling, have been successfully applied for treatment for RA and PsA (Sfikakis, 2010). Studies into the effects of such pharmaceuticals in neurodegenerative disorders like AD, PD and MS however remain limited.

Drugs targeting TNF-α for systemic inflammatory diseases still have adverse effects in a minority of patients (Hyrich et al., 2007). For instance, anti-TNF-α therapy for RA induces the risk of serious infections of the skin, soft tissues and joints (Atzeni et al., 2015). Anti-TNF-α agents increase risk rates of malignancies in patients with inflammatory bowel disease (Beigel et al., 2014). It has been mentioned that two patients with rheumatoid arthritis treated with anti-TNF-α strategy developed neurological symptoms, including demyelination lesions (Richez et al., 2005). Furthermore, it was shown that new-borns

presented severe neutropenia after their mothers were treated with infliximab for ulcerative colitis during pregnancy (Guiddir et al., 2014).

To reduce or diminish the drawbacks of anti-TNF-α agents in inflammatory diseases and neurodegenerative disorders, other therapeutic strategies should be investigated. Considering the various outcomes of TNF-α, targeting a specific point in its signalling pathways could be more effective and decrease the negative effects associated with anti-TNF-α agents.

2.6.2 Targeting TNFRs as Treatment for Neurodegenerative Disorders

In essence, TNFR1 has often been demonstrated to deteriorate or aggravate neurodegeneration whereas TNFR2 mediates neuroprotection. Therapeutics that specifically modulate the signalling mechanisms of TNF-α, *i.e.*, blocking TNFR1 actions and/or increasing TNFR2 signalling pathway could greatly reduce the side effects of current anti-TNF-α approaches. Sedger's group (Sedger et al., 2006) discovered that a leporipoxvirus TNF receptor homolog by its N-terminal preligand assembly domain (PLAD)-homologous domain interacts with the intracellular domain of TNFR1 and showed that this interaction results in a heterocomplex that inhibits TNFR1 downstream signalling and significantly prevents TNFR1-induced apoptosis of lymphocytes. Selectively blocking the bioactivity of sTNF-α, thus preventing TNFR1-mediated signalling, attenuated the pathological symptoms in EAE mice (Brambilla et al., 2011; Taoufik et al., 2011). Furthermore, a soluble TNFR1-selective antagonistic mutant TNF (named R1antTNF) ameliorated the symptoms in EAE mice (Nomura et al., 2011; Shibata et al., 2008). Currently, an antagonistic TNFR1 specific antibody has been produced and demonstrated to treat MS clinical symptoms more efficiently in EAE mouse model (Williams et al., 2014). Another human TNFR1 specific antagonistic antibody that may prove to have therapeutic effects is ATROSAB (Richter et al., 2013; Zettlitz et al., 2010).

As TNFR1 is mainly activated by sTNF-α, selectively inhibiting sTNF-α may prevent TNFR1-mediated apoptosis and could be a therapeutic strategy in neurodegenerative disorders. In this regard, a blocker of sTNF-α, XPro-1595, significantly improved the cognitive deficits induced by spinal cord injury in mice compared to the drug etanercept (Novrup et al., 2014). Furthermore, Tansey et al. (Barnum et al., 2014b) discovered that XPro-1595 significantly reduced activation of microglial and astrocytes, and prevented loss of dopamine neurons in a rat model of PD. Shedding of TNFR1 mediated by iNOS-cGMP-TACE signalling has been suggested to significantly ameliorate the inflammation associated with sepsis (Deng et al., 2015). Increased sTNFR1 levels resulting from this TNFR1 shedding could compete to bind to sTNF-α and impair TNFR1-mediated downstream signalling pathways and potentially reduce its apoptotic signalling pathways.

TNFR2-mediated signalling could be used as a therapeutic approach for neurodegenerative disorders. Lovastatin that is widely used to reduce cholesterol levels in patients has been confirmed to selectively increase TNFR2 expression (Nübel et al., 2005). Thereafter it was demonstrated that lovastatin protected primary cortical neurons against

glutamate-induced excitotoxicity (Dolga et al., 2008b). Moreover, *in vivo* evidence showed that lovastatin attenuated NMDA-induced nucleus basalis magnocelullaris (NBM) lesions and prevented cognitive deficits in mice (Dolga et al., 2009). This further supports the idea that TNFR2 activation could be a therapeutic approach for neurodegenerative disorders. Notably, Pfizenmaier's group (Fischer et al., 2011) constructed a soluble human TNFR2-selective agonist (TNC-scTNF$_{R2}$) and demonstrated that it successfully rescues human neurons from oxidative stress-induced cell death. TNC-scTNF$_{R2}$ is synthesized by genetic fusion of the trimerization domain of tenascin C to a TNFR2-selective signal-chain TNF molecule, which specifically activates TNFR2 to promote PI3K-PKB/Akt-NF-κB signalling pathway and promotes neuroprotection.

Selective targeting of TNFRs as a therapeutic strategy seems a promising avenue for the treatment of CNS conditions and some inflammatory diseases associated with TNF-α. Of note, it should be established whether simultaneous targeting of both receptors is necessary to achieve maximum therapeutic efficacy, as compared to inhibition/stimulation of either TNFR1 or TNFR2. Moreover, the ability of therapeutic compounds to cross the blood-brain barrier (BBB) is an obstacle that needs urgent attention. TNF-α blockers like etanercept and infliximab are too large to penetrate the BBB, and of certain new compounds it is yet to be examined whether they can pass the BBB. In the EAE mouse model, intraperitoneal injections with XPro-1595 and R1antTNF were effective in reducing pathology (Brambilla et al., 2011; Nomura et al., 2011; Taoufik et al., 2011). However, seeing the involvement of the peripheral immune system (and possibly the peripheral nervous system) in MS and EAE, it may be that targeting peripheral TNF-α is sufficient to reach therapeutic effects. Moreover, since the BBB is compromised in the EAE model, a possibility exists that these compounds may have entered the brain through an already leaky BBB. Nevertheless, a recent study on XPro-1595 in the 6-OHDA model for PD (in which the BBB is presumably not damaged enough to let XPro-1595 pass non-selectively) revealed that this compound could indeed reach the brain in therapeutically relevant concentrations (evidenced by inhibited glial activation and reduced dopamine neuron loss), after subcutaneous administration (Barnum et al., 2014b). Moreover, it may be that certain compounds, in certain conditions, do not necessarily have to pass the BBB to reach therapeutic effects. For example, in rat MCAO models for cerebral ischemia beneficial effects were reported upon intraperitoneal injection of etanercept (Arango-Dávila et al., 2014; Wu et al., 2015). Yet, it may be that etanercept could enter the brain via disruptions in the BBB, induced by the MCAO (Wu et al., 2015). Other important issues that will need to be addressed include the timing of treatment, and the potential side-effects that may arise from targeting TNF-α, TNFR1 and/or TNFR2. At least for ischemic stroke, start of treatment within a few hours after the ischemic insult may be crucial to limit damage as much as possible. However, as described in Chapter 3.3 of this review, the timing of specific TNF-treatments may be particularly delicate in acute conditions such as ischemic stroke, where TNFR expression levels may be rather dynamic in the first hours/days after the stroke. It may be that apoptotic signalling is beneficial for a certain length of time right after the insult in order to clear damaged cells and protect surrounding cells. As such, administering e.g. TNFR1 antagonists may have to be very carefully timed; neither too early nor too late. For chronic disorders like AD, PD and MS it can be hypothesized that beginning treatment in early stages may in the end prove to be most effective, but that

also starting treatment in later stages may well slow down neurodegenerative processes. Furthermore, the potential side-effects TNF-targeting compounds have to be assessed. For example, the possibility that selective inhibition of TNFR1 or stimulation of TNFR2 signalling could negatively affect production of neurotrophic factors (such as NGF and BDNF) should be explored.

2.7 CONCLUSIONS

As discussed in this review, TNF-α is involved in many neurodegenerative disorders by exerting both neuroprotective and neurodegenerative functions. A balance between these opposite effects seems to depend on its actions via TNFR1 and TNFR2. A thorough understanding of TNF-α signalling pathways can contribute to the development of potential therapeutic strategies. Focusing on multiple molecular interactions, which can control signalling outcomes in TNF-α signalling pathways could be critical to develop preferable therapeutic strategies in the future.

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Chapter 3

Generation, Identification and Characterization of Knock-in Mice with Chimeric Humanized TNFRs

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ABSTRACT

The role of tumor necrosis factor alpha (TNF-α) and its two receptors (TNFR1, TNFR2) has been implicated in the neuropathology of neurodegenerative diseases in animal models. Targeting TNF-α receptors could potentially be used to treat neurodegenerative diseases, such as MS, AD and stroke. The therapeutic potential of human TNFR-specific target agents for neurodegenerative disorders can only be properly studied in an *in vivo* model featuring human TNFRs. Mice carrying humanized TNFR represent an *in vivo* research model with human features. Here, we generated humanized TNFR1 and humanized TNFR2 knock-in mouse models in which the extracellular domains of mouse TNFRs were substituted by human counterparts, respectively. In this chapter, we describe the generation, identification and characterization of two humanized TNFR knock-in mice.

3.1 INTRODUCTION

Tumor necrosis factor alpha (TNF-α) has been implicated to play a complex role in neurodegeneration, in particular Alzheimer's disease (AD) and multiple sclerosis (MS) (Dong et al., 2015; Probert, 2015). TNF-α has been demonstrated to exert multiple biological activities due to the antithetic actions of its two receptors in cell lines and animal models: TNF-α binding to its receptor one (TNFR1) predominantly promotes inflammatory signalling pathways, which under certain circumstances leads to cell death (Fujita et al., 2016; Kälble et al., 2016), whereas TNFR2 signalling pathway mediates immune modulatory functions and promotes cell regeneration and survival (Chopra et al., 2016; Pozniak et al., 2016). In TNFR1 or TNFR2 knock-out models, a number of studies have already examined the roles of TNFR1 signalling pathway and TNFR2 signalling pathway. For instance, deletion of TNFR1 significantly diminishes Aβ formation and prevents learning and memory deficits (He et al., 2007); And deletion of TNFR2 in C57BL/6J mice was shown to lead to a severe deterioration in retinal ischemia (Fontaine et al., 2002). These data indicates that targeting TNFRs by inhibiting TNFR1 and/or activating TNFR2 could ameliorate pathophysiological traits of neurodegenerative disorders. Recently, TNFR1 blockade has been demonstrated to have therapeutic functions in a stroke model of mice (Liguz-Lecznar et al., 2015) and in an EAE model of mice (Williams et al., 2014). Moreover, another study showed that a selective human TNFR2 agonist rescued human neurons from oxidative stress-induced neuronal cell death *in vitro* (Fischer et al., 2011). These results suggest that TNFR1 blockade and TNFR2 agonists could be developed into potential treatment approaches against neurodegenerative disorders, including MS, AD and ischemic stroke. The therapeutic potential of targeting TNFRs agents for human neurodegenerative disorders can only be properly studied in an *in vivo* model featuring human TNFRs.

Humanized mouse models are an important prerequisite to develop experimental animal models to mimic human pathological conditions and to test compound efficacy. Humanized mouse models means that a mouse gene has been replaced by a portion of or by the entire human gene, and thereby the human proteins/domains are then expressed while the mouse protein/domain is suppressed in all cells and tissues of mice. Humanized mouse models have been applied to investigate various human diseases and disease therapeutics, including infectious diseases (Abraham et al., 2016; Ernst, 2016; Sandal et al., 2016), cancer (Hasegawa et al., 2016; Morton et al., 2016; Radin et al., 2016), injury-inflammation diseases (Carpenter et al., 2015; Hidalgo et al., 2009), immunotherapy of diseases (Lemmermann and Reddehase, 2016; Smith et al., 2016) as well as neurodegenerative diseases (Sevigny et al., 2016). For instance, the Sandrock group (Sevigny et al., 2016) has found that a human monoclonal antibody that selectively targets aggregated Aβ reduces Aβ plaques in Alzheimer's disease in a humanized mouse model. Therefore, humanized mice featuring human TNFRs are a useful research tool to investigate the features of human TNF/TNFRs signalling pathways *in vivo* and the therapeutic potential of human TNFR-specific targets. The establishment of humanized TNFRs mice by the use of knock-in technology has not been reported before.

In the present work, we used conventional molecular biological techniques to generate humanized TNFR knock-in mouse lines in which the extracellular domains of mouse TNFRs were substituted by human counterparts by homologous recombination, respectively. We described the generation and identification and characterization of humanized TNFR1 and humanized TNFR2 knock-in mice.

3.2 MATERIALS AND METHODS

3.2.1 Setup of Humanized TNFR Knock-in Mice

The humanized Tnfrsf1a and Tnfrsf1b knock-in mouse lines (hu/mTNFR1-k/i and hu/mTNFR2-k/i) were generated as contracted by Ozgene Pty Ltd (Bentley WA, Australia). For targeting TNFR1 the human sequence from chromosome 12 position 6330763 to 6332522 was inserted in place of mouse chromosome 6 positions 125306866 to 125310626. For TNFR2 the human sequence from chromosome 1 position 12188610 to 12193082 was inserted in place of mouse chromosome 4 positions 144814170 to 144819194. The targeting constructs were electroporated into C57BL/6J embryonic stem (ES) cell line, Bruce4. (Köntgen et al., 1993). Homologous recombinant ES cell clones were identified by Southern hybridization and injected into BALB/c-albino C57BL/6J blastocysts. Male chimeric mice were obtained and crossed to albino C57BL/6J females to establish heterozygous germline offspring on C57BL/6J background. The germline mice were crossed to a ubiquitous Cre mouse line to remove the loxP flanked selectable marker cassette.

To initially distinguish between hetero- and homozygous mice, the genotype of hu/mTNFR-transgenic mice was tested using mouse TNFR specific forward primers (mTNFR1 OZ fwd& mTNFR2 OZ fwd, Table 3.1) in combination with mouse or human specific reverse primers (mTNFR1 OZ rev, huTNFR1 OZ rev, mTNFR2 OZ rev & huTNFR2 OZ rev, Table 3.1). The mouse/mouse TNFR1 specific primers for wildtype TNFR1 lead to a product of 1217 bp, whereas the mouse/human TNFR1 specific primers for the chimeric TNFR1 lead to a product of 702 bp. Similarly, the mouse/mouse TNFR2 specific primers for wildtype TNFR2 lead to a product of 1169 bp, whereas the mouse/human TNFR2 specific primers for the chimeric TNFR2 lead to a product of 771 bp. After homozygous chimeric mice were bred for more than three generations, genotyping was carried out by PCR with primers specific for huTNFR1 (Table 3.2), leading to a 313 bp product for the human TNFR1 allele, and mTNFR1 (Table 3.2) inducing a 270 bp fragment for the wild type allele. Homozygous hu/mTNFR2-k/i animals were genotyped by PCR, using primers specific for huTNFR2 (Table 3.2) to produce a 300 bp fragment for human TNFR2 allele and using primers for mTNFR2 (Table 3.2) which results in 380 bp fragments for the wild type allele.

Table 3.1. Oligonucleotide sequences used for genotyping.

Primer	Sequence
mTNFR1 OZ fwd	5’-CTAAACATTCCTTGACCGGC-3’
mTNFR1 OZ rev	5’-TTCCCACACAAATCTTGACG-3’
huTNFR1 OZ rev	5’-ATGCTAGGGACAACAGCCAG-3’
mTNFR2 OZ fwd	5’-GGTCCAAACCTTCTAAGCCC-3’
mTNFR2 OZ rev	5’-ACATCAATATAGGCCAGCCG-3’
huTNFR2 OZ rev	5’-GCGTAGGGTGTAAATGCCAC-3’

Table 3.2.Oligonucleotide sequences used for PCR.

gene	forward	reverse
huTNFR1	5’-TGTCCCACCAAAACACACAC-3’	5’-CTGGCTGTTGTCCCTAGCAT-3’
mTNFR1	5’-CGGCTTCTTTTGCTTGTTTC-3’	5’-ACCTTTCCGACATGTCTTGC-3’
huTNFR2	5’-CTGGACTTTGTGGGGACAGT-3’	5’-GACAGCTGGAAGCCAAAGAG-3’
mTNFR2	5’-AAGGACCAGAGGTCTCAGCA-3’	5’-GCAGGAACAGAGGAGACGAG-3’

Male homozygous hu/mTNFR-k/i C57BL/6J mice (12 weeks, 24-30 g) were used for experiments. All animals were individually housed with a free access to food and tap water and kept in an air-conditioning room (21 ± 2°C) with a 12/12 h light-dark circle

(lights on 7:00 a.m.). Animal care and treatment were carried out in accordance with the local Ethical Committee guidelines on the use of experimental animals at the University of Groningen, the Netherlands (DEC6523) and University of Stuttgart, Germany (35-9815.81-0350).

3.2.2 Southern Blot Analysis

Embryonic stem (ES) cell DNA was isolated, digested with restriction enzymes and analyzed on agarose gels. Specificity of digestion products was tested by Southern blotting: DNA was transferred to nylon membrane and hybridized with the 5' or 3' external probe. Membranes were washed and pattern of hybridization was visualized on an X-ray film by autoradiography.

Genomic DNA from lung, heart, liver, brain, thymus, kidney and skin tissue of both homozygous hu/mTNFR1-k/i and hu/mTNFR2-k/i mice, respectively, was isolated, digested with *BamHI* and blotted on nylon membrane and probed with the endogenous probes (enP) for TNFR1:

5'CACATCTACCTCTTCCTGACACTGCCTGATCTGTTGGTTTGGCTTCAGGTTCTCTGATGGGGTTG
GAAGTACCACTGACCTTAGGTGCTCCAAGCATTTCTTCTTCGGGGAAAGGAACCACACTTTCATGA
TTGGGAAGTTCTTATCATAACTAACCCTTCCTGTCACCCTGGAAGCCTCTGTGTGTCGTGAGGGGT
GGGGGTGCACCCCTGCCTGAGAGATTGCTGGTGTGCTTTCTGTGTGTGGCTTCTTGGGTCTATGGC
TGAGGCAAGGGGCTTCCTGGCCCGTGCAGCTGCTGTGCCGAGGAGGTAGCACTTCTAGTAACAGC
AGCTGACAGCAGGGTGCAAGCTGCCAGCCTCTTTCCAAACGGAGCTTTGGGGGTTGCAGAGCCCC
CAAAGGCAGCTGTGAGTCTAGGTGTTAGGTCTCTCCTGAATGTGATCTGATTGGTCAGTTGCTTCT
GCATCTGTCTTGAAGACCTCCGCTATCTTGACGTAACAACGCTATGCGAGGGGGGGAGGGGAGAT
GAAAGAAGGAAGGCTGAGGGAAACCCAAAACCGGGAGAGATCAAAGAGCAAGGTCTACATTGA
AACTAAGAGGGCTTTGCGCTCTCCATACTGAAGCTGTTTACATACAAGAAAGCCAGTTGGTTGCTA
TGAGCTTTGCCAAGATTATAAAGCCAGCCCTCTAGAATGCCCTGCTTTGGGACTCCTGGCACATCC
TCAGCTCCTGAATTGAGGGTCATCTTGAATCAGATCACCAATCTCTGGTCAACTCTAGTACATGAC
ACATTTGTCTTTGGTCTTACTTAATCCTTGTCGCGCCTGCATGCTCGGAGTTCTTCCTTTTGTTTCTG
TCCCGCAATG3'

and for TNFR2:

5'TCATCTGGGGTTACTGGGTGAGCATGAAGACAAGGCAGGGGGCTGGCTGTATTGGGTTGCTTA
TCCAGGGCACGGTGTGCACAGTCGCCTGAAGAGGGAAGCACTCTGTGTGGACTTTTGGCCAAAG
GCGCAAGAGGCAGCTAGGGTACTGTGGCAGGTCCAGAAGAGCCAAAGACTGTCCACAGCATAGA
ACCCCAGTCCCTTGAGTCATGGCTTCTGGTGGCTTTCAGCTGTTATCACTGAGTGGCTGAGCAAAG
AGAAACATGATAGGCTCAGAGGTCACTCAGTGCACAGCACTAGGCAAGGTAGGGTGATGCTCTCC
AGACACAGCTGGGGATGGGAAGGGGAAAGACAGAAAGCAGAGCCACTGCCCACAGATCATGGG
AGTTTGTGCAGTCTATACAGACCAAATGAGCCTAATAGTAAGGTCGGCATCTTCTCTGATTGGCTT
GTTTGCAGTGGCTTTTCAGAGACTGATCCATGCCTTTGCTGTGCTAATTGTTGGATATTTTATGTAT
CTCTCTTGCTCCCATAGAATTGAGTCAGGAACCGATGTCCAGAGAGATAGGGACACTTGATTGACT
ATGACCATACAGCTTCTGGATGGAAGAATAAGAAAAGAAGACCACATCTCTCTAACTCTCCACCTT

TATCTGAGGAGTCAGGGGATTCATTTGGGTTACTGGTTTAATAAACATTTATTAAGCTCCTGCTGA
CCATATGACGGCAGCTGCTGGTACAATCAAGAGTGCGGAGCAGCCTTCCTCTTTAACCTAGAACCA
CCCCTTGGTCATTTCCAGGTGACATCAGCAGAGGCTGCAAGGGTGTTGAGTGTGTGTTCACACCCT
CTTTGCTCTTGTAG3'.

DNA from tail biopsies of C57Bl/6J wild type mice was used as a control.

3.2.3 Primary Mouse Embryonic Fibroblasts

Brain and dark red organs were removed from embryonic (E14–16) hu/mTNFR1-k/i or hu/mTNFR2-k/i mice. Remaining tissue was minced and digested using trypsin-EDTA (Gibco) and 1% DNase I (Sigma) for 30 min at 37°C. Digestion was stopped by addition of fetal calf serum (FCS) and homogenates were centrifuged (5 min, 200 g). Digested tissue was triturated in culture medium (DMEM, 10% FCS, L-Glutamine, 1% (v/v) penicillin/streptomycin (P/S)) and plated in cell culture dishes. After one to two days, cells were frozen or used for experiments.

3.2.4 Immunoblot Whole Brain Samples

The whole brain protein samples were prepared as described previously (Marchetti et al., 2004). Protein concentration from brain tissues were quantified and adjusted to 1 mg/ml. Samples were incubated on ice for 20 min. 25 µl of 0.1% Nonidet P-40 was added for 2 min, and the lysates were centrifuged at 8000 g for 10 min at 4°C. The lysates were boiled for 5 min in Laemmli's sample buffer (2% SDS, 5% DTT), and proteins was separated by SDS-polyacrylamide gel electrophoresis. After transfer to a PVDF membranes (Millipore, Billerica, MA, USA), membranes were blocked for 1 h with 1% I-blocker (Tropix, Bedford, MA, USA) in TBS containing 0.0625% Tween 20 and subsequently incubated overnight with primary antibody at 4°C (anti-human TNFR1 was purchased from Hycult Biotech, H398; anti-human TNFR2 was from Abcam, MR2-1). Afterwards, the membranes were washed with TBS containing 0.0625% Tween 20 and incubated with the appropriate HPR-conjugated secondary antibody diluted to 1:5000 with TBS containing 0.0625% Tween 20 for 1 h. Proteins were detected by using enhanced chemiluminescence (Piece Biotechnology, Rockford, IL, USA). Hypoxanthine guanine phosphoryltransferase (HPRT) served as internal standard protein.

3.2.5 Statistics

Data are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) of n independent experiments. Normal distribution was analyzed by Shapiro-Wilk normally test. Statistical analyses were performed by Student's t-test or one-way analysis of variance (ANOVA), followed by a post-hoc multiple comparison Tukey test. * $p < 0.05$ (** $p < 0.01$; *** $p < 0.001$) was considered significant.

3.3 RESULTS

3.3.1 Humanized TNFR Knock-in Mouse Models

To investigate and evaluate the potential functions of reagents targeting tumor necrosis factor receptors (TNFR1 and TNFR2) for human disease studies, we established humanized TNFR1 or TNFR2 knock-in (hu/mTNFR-k/i) mouse models. In these mouse models, the extracellular domains of mice TNFRs are replaced by the counterparts of human TNFRs, respectively, as shown in figure 3.1.

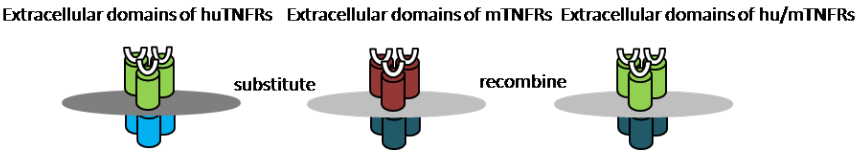

Figure 3.1. Humanized TNFRs knock-in mouse models. Schematic representation shows the structures of humanized TNFRs in chimeric mice.

DNA sequence that encodes extracellular domains of human TNFR1 and TNFR2 (described in materials and methods) was recombined into a targeting vector, the Ozgene plasmid PacL A90264BA-E12. By incorporating the targeted gene into the vector by homologous recombination, human TNFR1 or TNFR2 gene was introduced into the mouse gene locus (Figure 3.2A and 3.3A). The gene targeting constructs were electroporated into C57BL/6J embryonic stem (ES) cell line, Bruce4 (Köntgen et al., 1993). Homologous recombinant ES cell clones were identified by Southern hybridization and injected into BALB/c-albino C57BL/6J blastocysts. Male chimeric mice were obtained and crossed to albino C57BL/6J females to establish heterozygous germline offspring on C57BL/6J background. The germline mice were crossed to a ubiquitous Cre mouse line to remove the loxP flanked selectable marker cassette. Thereafter, the genotypes of hu/mTNFR1 and hu/mTNFR2 knock-in mice were determined by PCR.

The mouse genomic locus of TNFR1 could be digested by *EcoRV*, *BamHI,* or *NheI* to generate 11.9 kb, 3.8 kb, or 11.1 kb fragments, respectively (Figure 3.2 A). On the other hand, the targeted locus could be digested by *EcoRV, BamHI* or *NheI* to generate 7.7 kb, 7.7 kb or 7.5 kb fragments, respectively (Figure 3.2B). After the selectable cassette was removed, the gene locus of homozygous humanized TNFR1 mouse could be digested by *BamHI* to generate a 5.9 kb fragment (Figure 3.2C). Therefore, heterozygous and homozygous animals were distinguished via using Southern blot analysis using enzymes *EcoRV*, *BamHI* and *NheI* for digestion.

The hu/mTNFR2-k/i mouse models have been shown in Figure 3.3. In wildtype mice (Figure 3.3A), TNFR2 genomic locus contained *EcoRV*, *PshAI* and *BamHI* restriction enzyme sites, and these sites could digest TNFR2 genomic locus to be a 32.4 kb, a 26.7 kb and a 5.9

kb sequence, respectively. The targeted gene locus included the PGK-Neo-cassette and could be digested by *PshAI, BamHI* and *EcoRV* restriction enzymes (Figure 3.3B). The recombination gene locus could be digested by *PshAI* to generate a 13.5 kb band and a 14.5 kb band, and by *BamHI* to generate a 9.9 kb fragment. After the selectable cassette was removed, the genomic locus of chimeric mice could be digested by *BamHI* to generate a fragment of 8.2 kb (Figure 3.3C).

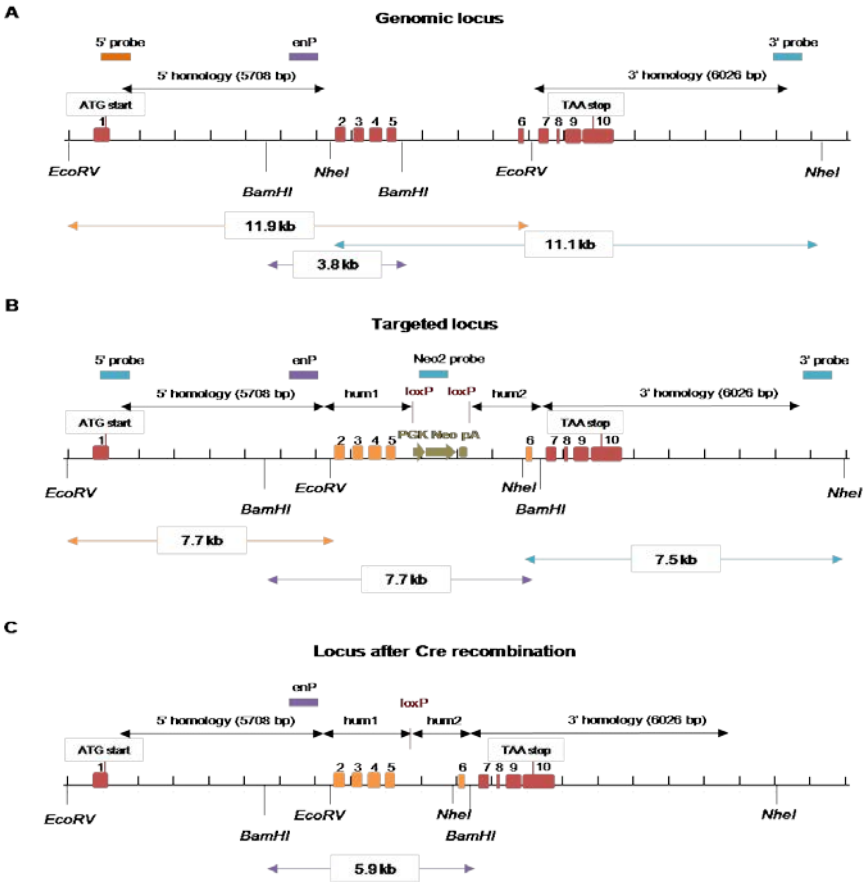

Figure 3.2. Homozygous humanized hu/mTNFR1-k/i mouse models. Schematic representation of the homologous recombination in the TNFR1 gene locus. Shown is the wildtype genomic locus (**A**), the targeted locus including the PGK-Neo cassette (**B**) and the genomic locus of chimeric mice (Cre'd locus, **C**). Heterozygous and homozygous animals were distinguished using southern blot analysis using enzymes *EcoRV*, *BamHI* and *NheI* for digestion. Detected products are annotated in the graph.

3.3.2 Screening and Identification of Humanized TNFR Knock-in Mice by Southern Blot

The targeted constructs of hu/mTNFR1 and hu/mTNFR2 were electroporated into C57BL/6J ES cell line, Bruce4 (Köntgen et al., 1993). Thereafter, nine homologous recombinant ES cells were identified by Southern hybridization (Figure 3.4). Genomic integration of hu/mTNFR1 was digested with *EcoRV* and probed with the 5' probe showed expected products with wildtype (wt) 11.9 kb and targeted 7.7 kb (Figure 3.4 A, left). Probing with the 3' probe and *NheI* digestion resulted in products of wt 11.1 kb and targeted 7.5 kb (Figure 3.4A, right). Genomic integration of hu/mTNFR2 was digested with *PshAI* and probed with the 5' probe that resulted in 26.7 kb fragments and targeted 13.5 kb fragments (Figure 3.4 B, left). Digestion with *EcoRV* and probing with the 3' probe resulted in products of wt 32.4 kb and targeted 17.8 kb (Figure 3.4B, right). Thereafter, we obtained recombinant ES cell clones of hu/mTNFR1 and hu/mTNFR2 that could be injected into BALB/c-albino C57BL/6 blastocysts.

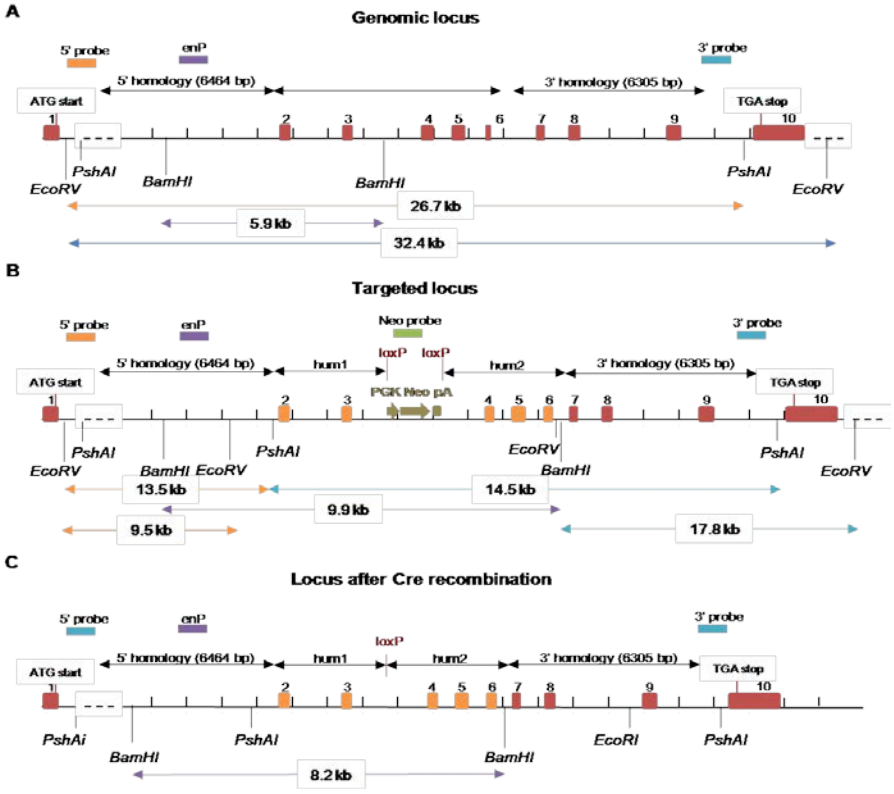

Figure 3.3. Homozygous humanized hu/mTNFR2-k/i mouse models. Schematic representation of the homologous recombination in the TNFR2 gene locus. Shown is the wildtype genomic locus (**A**), the targeted locus including the PGK-Neo cassette (**B**) and the genomic locus of chimeric mice (Cre'd locus, **C**). Heterozygous and homozygous animals were distinguished using southern blot analysis using enzymes *PshAI* and *BamHI* for digestion. Detected products are annotated in the graph.

Male chimeric mice were obtained and crossed to albino C57BL/6 females to establish heterozygous germline offspring on C57BL/6 background. The germline mice were crossed to a ubiquitous Cre mouse line to remove the loxP flanked selectable marker cassetteas shown in Figure 3.2C and Figure 3.3C. After the neo cassette was removed, DNA fragments of 5.9 kb and 8.2 kb DNA should be shown in homozygous hu/mTNFR1-k/i mice and homozygous hu/mTNFR2- k/i mice, respectively. Here, we analyzed DNA fragments of hu/mTNFR1 and hu/mTNFR2 in lung, heart, liver, brain, thymus, kidney and skin tissues of homozygous chimeric mice by Southern blot (Figure 3.4 C and D). Our data showed a 5.9 kb band in different tissues of hu/mTNFR2 k/i mice and a 3.8 kb band in wildtype mice (Figure 3.4 C); an 8.2 kb DNA band was shown in the different tissues of homozygous hu/mTNFR2-k/i mice, and a 5.9 kb DNA band was shown in wild type mice (Figure 3.4D).

3.3.3 Identification of Generation of Chimeric Mice

Upon removal of the neo cassette, the genotype of chimeric mice carrying hu/mTNFR1 or hu/mTNFR2 genes were tested by PCR with specific primers shown in Table 3.2. Homozygous and heterozygous hu/mTNFR1-k/i mice led to a 313 bp product for the human TNFR1 allele, and an approximate 270 bp fragment for the wildtype allele (Figure 3.5A). Mice that only showed a band at 313 bp were homozygous for hu/mTNFR1. Heterozygous mice presented bands at both 313 bp and at 270 bp, and only a band at 270 bp represented wildtype mice. Homozygous and heterozygous hu/mTNFR2-k/i mice produced a 380 bp fragment for human TNFR2 allele and a band at 300 bp for the wildtype allele (Figure 3.5B). Similar to the hu/mTNFR1-k/i mice, homozygous hu/mTNFR2-k/i mice only showed a band at 380 bp level; heterozygous mice presented bands at 380 bp and 300 bp, and only a 300-bp band represented wildtype mice.

3.3.4 Hu/m TNFR1 and Hu/m TNFR2 Expression in Chimeric Mice

The expression levels of hu/mTNFRs in primary mouse embryonic fibroblasts (MEFs) were also analyzed by flow cytometry (Figure 3.6 A and B, were performed by our collaborator, Roman Fischer, Germany). We found that hu/mTNFR1 and hu/mTNFR2 were stably expressed in chimeric mice and that the levels of hu/mTNFR1 and hu/mTNFR2 expression in chimeric mice were similar with levels of TNFR1 and TNFR2 expression in wildtype mice. Moreover, we isolated the brain tissue and tested hu/mTNFRs expression in hu/mTNFR1 k/i, hu/mTNFR2 k/i and wildtype mice by Western blot (Figure 3.6 C and D). Furthermore, our collaborator (Roman Fischer) also tested the expression of hu/mTNFR1 and hu/mTNFR2 in chimeric homozygous mice using flow cytometry to analyze plenocytes and thymocytes (Supplementary figure 3.1). The comparison with wildtype mice showed that TNF receptors were expressed at similar levels.

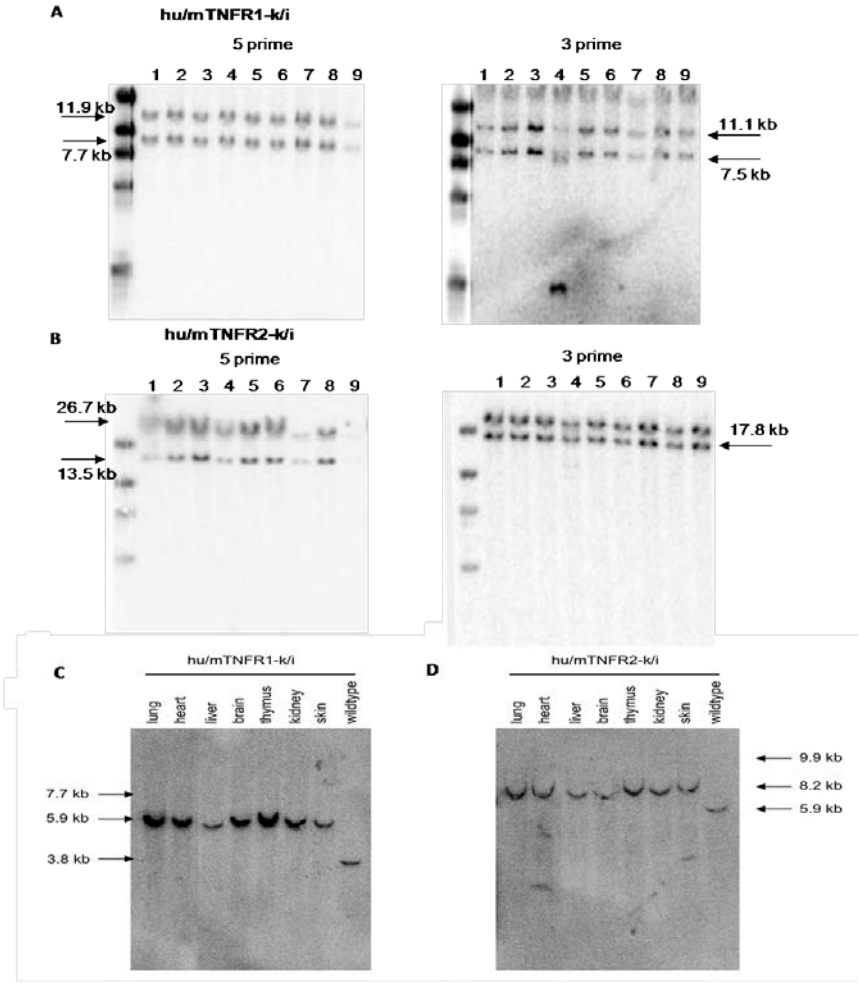

Figure 3.4. Southern blot analysis of ES cells and mouse tissues. (**A**) Genomic integration of hu/mTNFRswas shown for nine embryonic stem cells. DNA was digested with *EcoRV* and probed with the 5' probe, andresulted in 11.9-kb DNA size (wildtype, wt) and a 7.7 kb DNA size (targeting, left); Probing with the 3' probe and *NheI* digestion resulted in products of wt 11.1 kb and targeted 7.5 kb (right). (**B**) Genomic integration of hu/mTNFR2 was shown for nine embryonic stem cells. DNA digested with *PshAI* and the 5' probe showed wt 26.7 kb and targeted 13.5 kb (left); digestion with *EcoRV* and probing with the 3' probe resulted in products of wt 32.4 kb and 17.8 kb (targeted, right). DNA from lung, heart, liver, brain, thymus, kidney and skin tissue from homozygous hu/mTNFR1-k/i (**C**), hu/mTNFR2-k/i (**D**) mice were digested by *BamHI*, blotted on nylon membrane and probed with the endogenous probes enP. The DNA size marker phage Lambda DNA/StyI marker (Bioron, Ludwigshafen, Germany) was used to identify size of products. In all samples of homozygous

transgenic mice the neo cassette was removed as evident by the 5.9 kb (A, hu/mTNFR1-k/i) or 8.2 kb (B, hu/TNFR2-k/i) product.

3.4 DISCUSSION

Since tumor necrosis factor (TNF) and its two receptors (TNFR1 and TNFR2) have been reported to play a predominant role in the initiation and orchestration of immunity and inflammation and neurodegeneration (Aggarwal, 2003; Dong et al., 2015; Karatas et al., 2016; Murayama et al., 2015), chemicals that target TNFRs could be developed into therapeutic drugs. It implies that establishment of humanized TNFRs mice is necessary for the investigations of human TNFR targets to treat certain diseases such as AD. Here, we generated two humanized mouse models in which the extracellular domains of mouse TNFRs were replaced by the human TNFRs counterparts, respectively. The design strategy on the two humanized TNFR mouse models was that mouse extracellular domains were replaced by human extracellular domains and mouse intracellular domains still maintain in the chimeric mice. Advantages of this design strategy are that in the two humanized mouse models the constructs of humanized TNFRs not only can be modulated by human TNFRs specific agonists or antagonists but also maintainthe downstream signalling pathways in the chimeric mice.

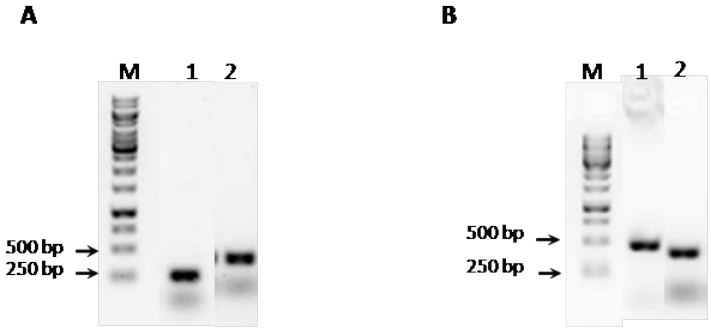

Figure 3.5. The genotypes of hu/mTNFR1 and hu/mTNFR2 knock-in mice were determined by PCR. (**A**) The size of the humanized TNFR1 PCR product was 313 bp (lane 2), and that of mouse TNFR1 was approximately 270 bp (lane 1). (**B**) The size of humanized TNFR2 PCR product was shown a band at 380 bp and mouse TNFR2 was shown at 300 bp. Lane M represents a 1 kb DNA ladder.

On the other hand, one previous study had demonstrated that transgenic mice expressing 3'-modified human TNF-α showed deregulated patterns of expression and developed chronic inflammatory polyarthritis (Butler et al., 1997; Keffer et al., 1991). Moreover, TG197hTNFR1 knock-in mouse model carrying fully human TNF-TNFR1 genes spontaneously develop arthritis due to the interaction of overexpressed human TNF with human TNFR1 (*http://www.biomedcode.com/gr/en/content/human-tnf-tnfr1-driven-arthritis*) (Michopoulos et al., 2016). However, we just sought to determine therapeutic effects of human TNFR-specific reagents against neurodegeneration in certain humanized

TNF or TNFR transgenic mouse models with normal phenotypes. Humanized TNF-α transgenic mouse models that can spontaneously develop inflammation diseases were not reasonable models for investigations of TNFR-specific reagents against neurodegeneration. Therefore, we established two novel humanized TNFR knock-in (hu/mTNFR-k/i) mouse models expressing extracellular domains of human TNFR1 or human TNFR2 that could respond human TNFR-specific reagents, respectively. Notably, we found that the two humanized mouse models show normal physiological features but no signs of inflammatory diseases, such as arthritis.

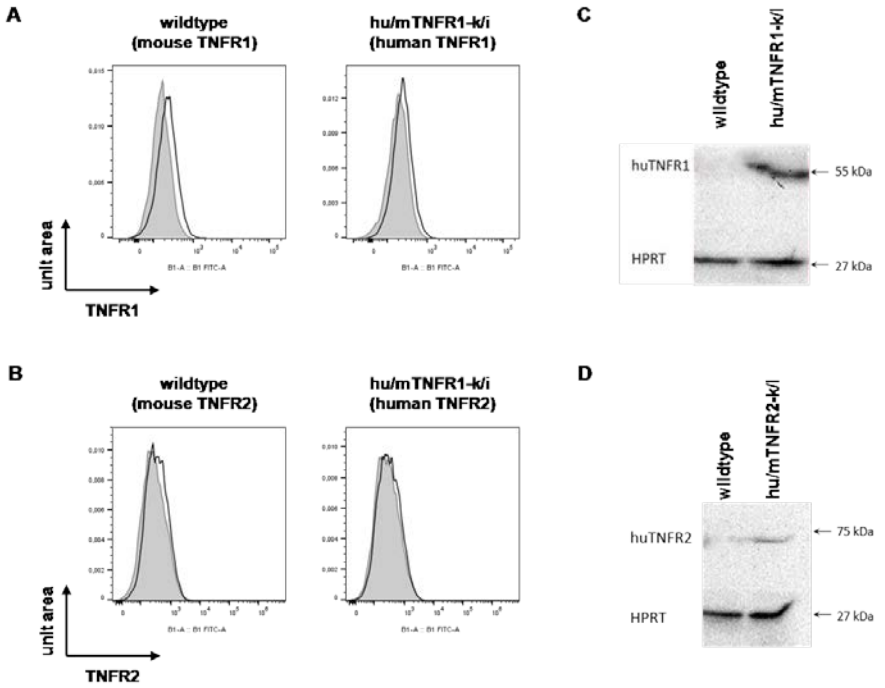

Figure 3.6. Expression of transgenic hu/mTNFR1-k/i and hu/mTNFR2-k/i in primary cells isolated from chimeric mice. Primary mouse embryonic fibroblasts (MEF) were isolated from wildtype (**A**, **B**), hu/mTNFR1-k/i (A) or hu/mTNFR2-k/i (B) C57BL/6 mice. Expression o f mouse TNFR1 (HP8002) and human TNFR1 (HP9002) (A) or mouse TNFR2 (HP8003) and human TNFR2 (HP9003) (B) was analyzed by flow cytometry (performedby Roman Fischer). Data are presented as normalized to unit area. (**C**, **D**) Brain tissueisolated from wildtype (C,D) hu/mTNFR1-k/i (C) or hu/mTNFR2-k/i (D) C57BL/6 mice. Tissue was lyzed and expression of huTNFR1 (wildtype, hu/mTNFR1-k/I,H398) or huTNFR2 (wildtype, hu/mTNFR2-k/I,MR2-1) was analyzed by western blot.

In the present work, we found that the chimeric TNFRs genes are present in many tissues including lung, heart, liver, brain, thymus, kidney and skin by Southern blotanalysis (Figure

3.4 C and D). Chimeric TNFRs can expressstably in the brain tissue of chimeric mice by Western blot analysis (Figure 3.6 C and D), and the levels of chimeric TNFRs are similar with the levels of mouse TNFRs in wildtype mice (Figure 3.6 A and B, the flow cytometry analyses were performed by Roman Fischer, Germany). Additionally, we also found that chimeric TNFRs can be activated by mouse TNF-α as expected (Supplementary figure 3.2). Thus, the humanized TNFRs mouse models can be used for studies of human TNFRs signalling pathways *in vivo*, and investigations of therapeutic potential of specific drugs for inflammatory diseases and neurodegenerative disorders such as multiple sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD).

Humanized mouse models are critical tools for basic research, modeling of certain human-specific diseases, efficacy testing of immunotherapy approaches, and safety assessment for some molecule therapeutics (Goettel et al., 2016; Grün et al., 2016; Lundbäck et al., 2016; Masuda et al., 2016; Mukherjee et al., 2016; Radin et al., 2016). Humanized mice as a model had been used to study human hematopoietic stem cell transplantation (Denton et al., 2012). Humanized mice were also applied to study graft-versus-host disease during allogeneic bone marrow transplantation (BMT) (Zheng et al., 2013). Additionally, application of mice with human immune system was investigated for HIV drug discovery (Ibeh et al., 2016). We here generated two humanized mouse models featuring human TNFR1 and human TNFR2. Generation of the two humanized TNFR mouse models represents a major advance in current TNFRs research models compared to *in vitro* systems or non-human primate models. Dependent on the multiple roles of TNFRs in certain diseases, the two humanized mouse models featuring human TNFR1/2 can be applied to study the therapeutic potential of human TNFR-specific reagents. For instance, the roles of TNFRs have been reported in neurodegenerative disorders (Dong et al., 2015; Kollias and Kontoyiannis, 2002), such as AD and MS, thus human TNFR-specific reagents could be applied to treat these diseases in hu/mTNFR-k/i mice.

In summary, the present work showed development and characterization of the humanized TNFR1 and humanized TNFR2 knock-in mouse models and provided evidence that the recombined TNFRs can be expressed stably in chimeric mice, indicating a more broadinvestigation of human TNF systems *in vivo*. We expect that using transgenic mouse models will facilitate the development of agents for translational sciences and therapeutics.

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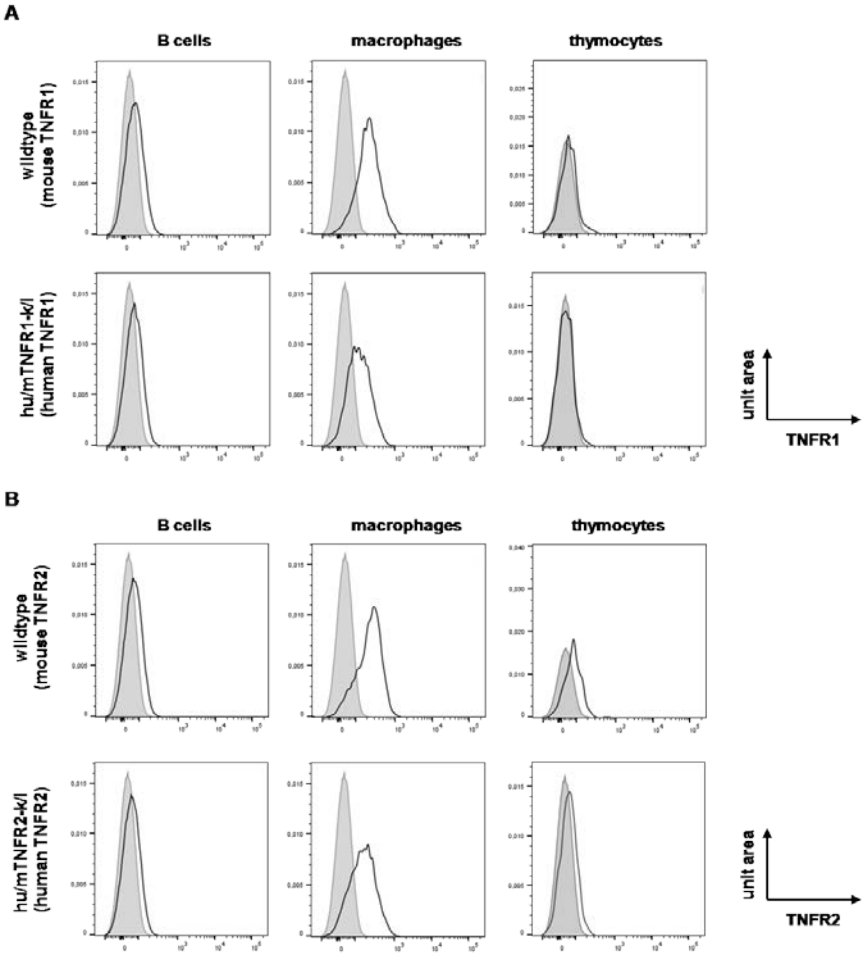
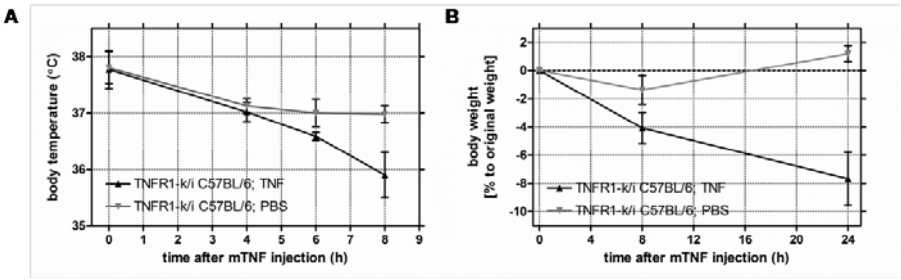
SUPPLEMENTARY MATERIALS AND RESULTS

Isolation of Thymocytes and Splenocytes for Flow Cytometry

Thymus and spleen of hu/mTNFR1-k/i or hu/mTNFR2-k/i mice were isolated and mashed through a 40 µm cell strainer (Falcon). Cells were centrifuged (300 g, 5 min) and washed once with PBA (PBS, 0.5% BSA, 0.02% NaN_3). Then 0.5 x 10^6 cells were incubated with antibodies against mouse TNFR1 (HP8002) and human TNFR1 (HP9002) and mouse TNFR2 (HP8003) and human TNFR2 (HP9003, Hycult Biotec, Uden, The Netherlands) for 45 min on ice. Cells were washed twice with PBA and incubated with fluorescence labeled secondary antibodies for 30 min on ice. Then cells were washed again twice with PBA and fluorescence was analyzed by flow cytometry (MACSQuant Analyzer 10, Miltenyi), Normalized data are presented as unit area vs fluorescence intensity. Antibodies against CD45R, CD68 and CD8 were from Miltenyi Biotech (Bergisch-Gladbach, Germany). Secondary antibodies coupled to Alexa-Fluor488 were from Life Technologies (Karlsruhe, Germany) and horseradish peroxidase (HRP)-labeled antibodies were purchased by Jackson ImmunoResearch Laboratories (Suffolk, UK).



Supplementary figure 3.1. Expression of transgenic hu/mTNFR1-k/i and hu/mTNFR2-k/i in chimeric homozygous mice. Splenocytes or thymocytes were isolated from C57BL/6J wildtype (**A**,**B**), hu/mTNFR1-k/i (A) or hu/mTNFR2-k/i (B) mice. Then mouse TNFR1 (HP8002) and human TNFR1 (HP9002) (A) or mouse TNFR2 (HP8003) and human TNFR2 (HP9003) (B) expression was analyzed by flow cytometry using sub-gates for CD45R+ B cells, CD68+ macrophages (splenocytes) and CD8+ thymocytes. Gray histograms show the isotype controls, black lines indicate signals measured for TNFR1 or TNFR2 expression. Data are presented as normalized to unit area (This was done by Roman Fischer).



Supplementary figure 3.2. TNF-induced cytotoxicity in hu/mTNFR1-k/i mice. hu/mTNFR1-k/i mice were treated with mouse TNF (30 µg) or saline (i.v.). Animals were monitored over a period of 24 hours and body temperature (**A**) and body weight (**B**) was documented (n = 4 - 6 mice per group, ± SEM).

Chapter 4

Selective Targeting Tumor Necrosis Factor Receptors: Essential Protective Role of TNFR2 in Neurodegeneration

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ABSTRACT

Tumor necrosis factor alpha (TNF-α) that is a major pro-inflammatory cytokine has been implicated to contribute to physiopathology of onset in a number of neurodegenerative disorders. Despite the recognized role of TNF-α in inflammation and neurodegeneration, anti-TNF-α reagents failed to treat neurodegenerative disorders. Animal disease models had revealed the antithetic effects of the two TNF receptors (TNFR1 and TNFR2) in the central nervous system, whereby TNFR1 has been associated with inflammatory degeneration and TNFR2 with neuroprotection. This indicates that selective activation of TNFR2 or/and inhibition of TNFR1 might be of therapeutic benefit for neurodegenerative diseases. Here, we showed the therapeutic potential of selective activation of TNFR2 by human TNFR2-selective agonists, EHD2-scTNF$_{R2}$ and TNC-scTNF$_{R2}$, and selective inhibition of TNFR1 by a human TNFR1-specific antagonistic antibody, ATROSAB, respectively, in a mouse model of N-methyl-D-aspartate (NMDA) induced nucleus basalis magnocellularis (NBM) lesion. We found that co-administration of TNC-scTNF$_{R2}$ or EHD2-scTNF$_{R2}$ or ATROSAB into the NBM significantly protected cholinergic neurons and their neocortical projections. Also, the reagents significantly prevented microglial activation at the site of injection in the brain. Moreover, we also found that simultaneous blocking TNFR1 and TNFR2 signalling, however, abrogated the therapeutic effect of ATROSAB, uncovering an important role of TNFR2 in neuroprotection. Accordingly the therapeutic activity of ATROSAB is mediated by shifting the balance of the antithetic activity of endogenous TNF-α towards TNFR2, which appears essential for neuroprotection. To conclude, our data indicate that selective targeting TNFRs could be effective therapeutic approaches for developments of drugs against neurodegenerative diseases in future.

4.1 INTRODUCTION

Tumor necrosis factor alpha (TNF-α) is one of the major pro-inflammatory cytokines that plays an important role in the initiation of immunity and inflammation (Aggarwal, 2003; Fischer and Maier, 2015). Elevated levels of TNF-α have been associated with different autoimmune diseases; deregulation of TNF-α expression and signalling can lead to chronic inflammation and tissue damage (Dong et al., 2015; Fischer et al., 2015; Kollias, 2005; Probert, 2015). Therefore, several anti-TNF-α therapeutics are clinically approved and used to treat autoimmune disease, such as rheumatoid arthritis, psoriasis or inflammatory bowel disease. Additionally, up-regulated TNF-α expression has also been associated with in a variety of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), stroke and Multiple Sclerosis (MS) (McAlpine et al., 2009; Tan et al., 1999; Tarkowski et al., 2000; Yang et al., 2013). However, an anti-TNF-α therapeutic (Lenercept) failed in a Phase II randomized, multi-center, placebo-controlled study for the treatment of relapsing remitting MS because symptoms of Lenercept-treated patients were significantly increased compared to patients receiving placebo and neurologic deficits tended to be more severe in the Lenercept treatment group study (1999). Next to induction or aggravation of demyelinating diseases, all anti-TNF-α therapeutics may induce severe side-effects such as serious infections, including reactivation of tuberculosis, invasive fungal and other opportunistic infections.

The failure of the Lenercept study and the severe side-effects of anti-TNF-α therapeutics might be explained by the pleiotropic functions of TNF-α, including both pro- and anti-inflammatory functions and other immune regulatory as well as regenerative activities, through its two receptors (TNFR1 and TNFR2). Blocking all effects of TNF-α therefore might be counter-productive in neurodegenerative disorders. Because most of the pro-inflammatory actions of TNF-α are mediated by TNFR1 (Fontaine et al., 2002; Maddahi et al., 2011; Yang et al., 2013; Zhao et al., 2003), a more effective therapeutic approach could be the selective blocking of TNFR1 signalling. Indeed, TNFR1 inhibition has been shown to significantly ameliorate experimental autoimmune encephalomyelitis (EAE) symptoms in a mouse model (Williams et al., 2014). This neuroprotective approach of TNFR1 inhibition could spare TNFR2 signalling, which has been implicated in various protective and regenerative responses, particularly in the central nervous system (CNS): TNFR2 signalling was shown to promote neuronal survival and oligodendrocyte regeneration in *in vivo* models of ischemic and neurotoxic insults (Arnett et al., 2001; Fontaine et al., 2002). Therefore, according to the antithetic effects of both TNF-α receptors, targeting TNFRs by inhibiting TNFR1 and/or activating TNFR2 signalling pathway might represent a potential therapeutic strategy against neurodegenerative disorders.

In the present study, we investigated the therapeutic potential of novel human TNFR2 selective agonists, TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$, and a novel human TNFR1 specific antagonist, ATROSAB, using a well-established model of *in vivo* brain damage, the nucleus basalis magnocellularis (NBM) lesion (Whitehouse et al., 1981;Coyle et al., 1983), in humanized TNFR knock-in mice (hu/mTNFR1-k/i and hu/mTNFR2-k/i) mice, respectively. We discovered that TNC-scTNF$_{R2}$, EHD2-scTNF$_{R2}$, and ATROSAB significantly prevent the

NBM lesion-induced cortical cholinergic denervation and massive size of microglial activation in both transgenic mouse lines. Also, we found that the neuroprotection of ATROSAB is mainly dependent on activation of TNFR2 signalling pathway.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Primers for gene expression studies were obtained from Biomers (Ulm, Germany) and for genotyping of humanized TNFR from Life Technologies. The TNFR2 specific antibody 80M2 (Grell et al., 1995) and the TNFR1 selective antagonistic antibody ATROSAB (Zettlitz et al., 2010) have been described. Antibodies against Akt and phospho-Akt (Ser473) were from Cell Signalling Technology (Boston, MA). The anti-TNF and anti-TNFR2 (MAB426) antibody was from R&D System (Wiesbaden, Germany). Secondary antibodies coupled to Alexa-Fluror488 were from Life Technologies (Karlsruhe, Germany) and horseradish peroxidase (HRP)-labeled antibodies were purchased by Jackson ImmunoResearch Laboratories (Suffolk, UK). Primary antibodies used for immunohistochemistry were goat anti-ChAT IgG (Millipore, Billerica, MA, USA) and mouse anti-rat integrin αM (CD11b) monoclonal antibody (Chemicon International, Temecula, CA, USA). Secondary antibodies were rabbit anti-goat IgG (Sigma) and horse anti-mouse IgG (Vector, Brunschwig Chemie, Amsterdam, the Netherlands), respectively. Stainings were developed by use of the Vectastain Elite ABC Kit (Vector Laboratories) followed by incubation with 3,3'-diaminobenzidine (DAB,Sigma, St. Louis, MO, USA). Actinomycin D and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

4.2.2 Animals

Male homozygous hu/mTNFR-k/i C57BL/6J mice (12 weeks, 24-30 g) were used for experiments. All animals were individually housed with a free access to *ad libitum* food and tap water and kept in an air-conditioning room (21 ± 2°C) with a 12/12 h light-dark circle (lights on 7:00 a.m.). Animal care and treatment were carried out in accordance with the local Ethical Committee guidelines on the use of experimental animals at the University of Groningen, the Netherlands (DEC6523) and University of Stuttgart, Germany (35-9815.81-0350).

4.2.3 Nucleus Basalis Magnocellularis Injection and Treated Groups

An injection into the NBM was performed as previously described by Luiten et al. (Luiten et al., 1995). Briefly, after anesthesia with isoflurane, mouse heads were mounted in a

Kopf stereotactic frame (Kopf Instruments model 900, Tujunga, CA, USA). A volume of 0.6 µl solution was slowly infused in two steps of 0.3 µl into the NBM of the right hemisphere (coordinates: 0.6 mm posterior to bregma; 2.1 mm lateral to the sagittal suture; 4.6/4.4 mm ventral to the dura; Paxinos and Franklin, 2001). After the injection (0.1 µl/min), the needle of a 1-µl Hamilton syringe was left *in situ* for 2 min to allow for an effective drug diffusion. The contralateral intact hemisphere served as internal control without interference. After 8 day upon the NBM injection, all mice were sacrificed.

Hu/mTNFR2-k/i mice were divided into 6 groups: (1) phosphate-buffered saline (PBS, 0.01 M, pH = 7.40); (2) PBS with 55 nmol NMDA (Sigma, USA); (3) PBS with 220 ng TNC-scTNF$_{R2}$ (Stuttgart, Germany); (4)NMDA with 220 ng TNC-scTNF$_{R2}$; (5) PBS with 580 ng EHD2-scTNF$_{R2}$ (Stuttgart, Germany); (6) 55 nmol NMDA together with 580 ng EHD2-scTNF$_{R2}$. Hu/mTNFR1-k/i mice were divided into 7 groups: (1) PBS; (2) PBS with 3 µg ATROSAB; (3) 55 nmol NMDA; (4) 55 nmol NMDA together with 3 µg ATROSAB; (5) 55 nmol NMDA, 3 µg ATROSAB and 150 ng mouse TNFR2 antagonist (MAB426); (6) 55 nmol NMDA together with 150 ng MAB426 only; (7) 55 nmol NMDA and 2 µg anti-human epidermal growth factor receptor (huEGFR) IgG as an unspecific control antibody.

4.2.4 Tissue Processing and Immunohistochemistry

Under a deep anesthesia via an i.p. injection of pentobarbital sodium, mice were processed by a short pre-rinse with 0.5% heparin physiological saline and subsequently transcardially perfused by using a fixative solution of 4% paraformaldehyde in phosphate-buffer (PB, 0.1 M, pH = 7.40). Brain tissues were removed, post-fixed 48 h with composition of 4% paraformaldehyde in PB, stored in PB at 4°C for 24 h, and cryoprotected by immersion in 30% sucrose at room temperature for 24 h. Afterwards, frozen coronal sections at a 20-µm thickness were cut on a cryostat microtome and collected in PBS (0.01 M, pH = 7.40) containing 0.1% sodium azide.

Projected cholinergic fibres After rinsing several times in PBS, free-floating tissue sections were processed for choline-acetyltransferase (ChAT) immunohistochemistry by incubation in PBS with 0.3% H_2O_2 for 45 min, rinsed several times again, and pre-incubation in PBS containing 5% normal rabbit serum (NRS, Zymed, San Francisco, CA,USA) and 0.4% Triton X-100 at room temperature (RT) for 1 h. Subsequently, sections were incubated with goat anti-ChAT IgG (diluted 1:333, Millipore, Billerica, MA, USA) in PBS containing 1% NRS, 0.5% BSA and 0.4% Triton X-100 at 4°C for 3 days. After incubation of the primary antibody, sections were rinsed in PBS and incubated with rabbit anti-goat IgG (diluted 1:500, Sigma) in PBS containing 1% NRS, 0.2% Triton X-100 and 0.5% BSA at RT for 4 h and at 4°C overnight. Sections were rinsed in PBS and incubated in PBS with Vectastain Elite ABC Kit (both A solution and B solution were diluted to 1:500) at RT for 2 h. Finally, 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) reaction to peroxidase as chromogen was visualized. Visualization of DAB reaction was enhanced by Ammonium nickel sulphate (BDH Chemicals Ltd., UK).

Microglial activation Tissue sections were incubated in 0.3% H_2O_2 in PBS for 45 min, rinsed in PBS several times, pre-incubated in 5% normal horse serum (NHS) and 0.4% Triton X-100 PBS. Thereafter, sections were incubated with mouse anti-rat integrin αM monoclonal antibody (CD11b, diluted 1:1000, Chemicon International, Temecula, CA, USA) in PBS containing 1% NHS and 0.4% triton X-100 at 4°C for 3 days. Sections thereafter were incubated in Biotinylated horse anti-mouse IgG (Vector, Brunschwig Chemie, Amsterdam, the Netherlands) diluted 1:500 at 4°C overnight, followed by incubation of Vectastain Elite ABC Kit (diluted to 1:500) at RT for 2 h. Thereafter, peroxidase was visualized by DAB as chromogen. Visualization of the DAB reaction was enhanced by Ammonium nickel sulphate.

4.2.5 Quantification of the NBM Lesions

Quantitative analysis of cholinergic fibres ChAT-positive innervation density was measured in the somatosensory cortex by using a program of the LAS Image Analysis Software (Leica Quantimet, the Netherlands). Surface area density of cortical ChAT-positive fibres was measured in the layer V of somatosensory cortex in eight coronal sections (coordinates: 0.6 mm posterior to bregma), which represent a strong reaction of cholinergic innervations from the lesioned NBM subdivision (Gaykema et al., 1990). After ground subtraction and gray-scale threshold determination, the surface area of ChAT-positive fibres in the layer V of the parietal cortex was calculated by shown as a percentage by (the area coverage of ChAT-active cholinergic fibres)/(the total sampling area) x 100 in each coronal section. The correlated value of fibre reduction caused by the unilateral NBM lesion was calculated as the percentage difference between the surface area density of cortical fibres in the damaged side and that in contralateral side (Harkany et al., 2001).

Measurement of microglial activation Quantification on the reactive volume of microglial activation has been previously described (Granic et al., 2010). Briefly, the size of the reactive region of CD11b-positive microglial activation was measured on the surface area of coronal sections in the lesion site by using a program of the Quantimet 600 system (Leica, the Netherlands). The reactive surface area of microglial activation was quantified in a series of CD11b-positive sections of each mouse brain. The total reactive volume of microglial activation in a brain was calculated as $(x_1 + x_2 + \ldots x_n) \times 100\ \mu m^3$, where '$x_n$' is the reactive surface area of each section (μm^2) and 100 μm was the distance between two consecutive sections.

4.2.6 Primary Cortical Neuron Culture

Primary cortical neurons were prepared from embryonic brains (E14–16) of hu/mTNFR1-k/i mice. The meninges were removed and the cortical neurons were separated by mechanical dissociation. Cells were plated in a density of 12×10^4 cells/well (96-well plates) and 2×10^6 cells/well (6-well plates) on poly-D-lysine pre-coated plates. Neurobasal medium (Gibco) with 2% (v/v) B27-supplement, 0.5 mM glutamine, 1% (v/v)

penicillin/streptomycin was used as culture medium. After 48 h neurons were treated with 10 µM cytosine arabinoside (Sigma) for another 66 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and after 7 days of in vitro culture, the neurons were used for experiments.

4.2.7 MTT Assay

Cells were incubated with MTT (0.5 mg/ml) for 2 h at 37°C. Then lysis buffer (10% SDS, 20 nM HCl) was added, cells were lyzed overnight and optical density at 550 nm was determined. Each sample was analyzed in triplicates and data were analyzed using the software Microsoft Excel and GraphPad Prism 4.

4.2.8 Western Blot

Cells were lysed in homogenization buffer (10 mM HEPES pH 7.5, 1.5 mM MgCl2, 1.5 mM KCl, 1% NP-40, 0.2 mM PMSF, 20 mM β-glycerophosphate and 100 µM Na_3VO_4) at 4°C for 30 min. Lysates were centrifuged (2 min at 9600 g) and protein concentration of supernatants were determined using the BCA method (Pierce, Bonn, Germany). 20 µg total proteins were denatured in Laemmli buffer and resolved by 12% SDS-PAGE (100 V; 90 min). Then, proteins were transferred onto nitrocellulose membranes (semidry blot; 1.5 mA/cm^2 gel for 90 min) and non-specific protein binding was blocked with 5% skimmed milk powder solution in PBS with 0.1% Tween 20 for 30 min at RT and the membrane was incubated overnight at 4°C using specific antibodies. After incubation with HRP-conjugated secondary antibodies for 90 min at RT the signals were detected by enhanced chemiluminescence (Super Signal, Pierce, Rockford, IL).

4.2.9 Statistics

Data are presented as mean ± standard error of the mean (SEM) of n independent experiments. Statistical analyses were performed by Student's t-test or ANOVA, followed by a post-hoc Tukey range test. * $p < 0.05$ (** $p < 0.01$; *** $p < 0.001$) was considered significant.

4.3 RESULTS

4.3.1 TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$ Prevent the NBM Lesion-induced Cholinergic Denervation and Neuroinflammation in hu/mTNFR2-k/i Mice

To assess the potential effects of both EHD2-scTNF$_{R2}$ and TNC-scTNF$_{R2}$ in an *in vivo* model of neurodegeneration, hu/mTNFR2 knock-in mice were used to establish the nucleus basalis lesion model in which NMDA was stereotactically injected into the nucleus basalis

magnocellularis (NBM). Our data showed that lesioning of the NBM resulted in a reduction of body weight (Figure 4.1), as compared to PBS-injected group. Application of TNC-scTNF$_{R2}$ or EHD2-scTNF$_{R2}$ together with NMDA injected into the NBM showed a significant prevention for the loss of body weight (Figure 4.1, $p < 0.01$).

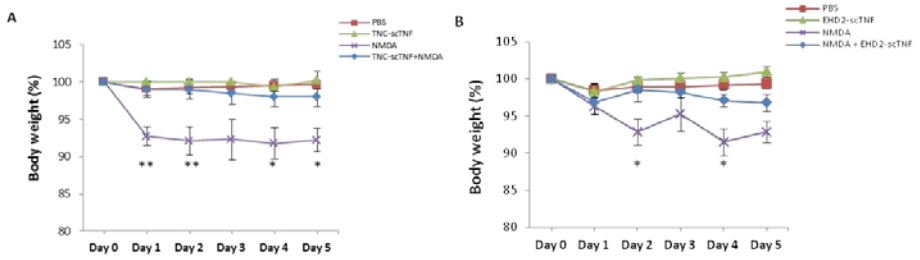

Figure 4.1. Body weight changes after pharmacological treatment in hu/mTNFR2-k/i mice. (**A**) TNC-scTNF$_{R2}$ prevented the significant reduction of body weight induced by NMDA injection into the NBM after injection 1 day. (**B**) EHD2-scTNF$_{R2}$ significantly prevented the reduction of body weight induced by NMDA injection into the NBM in hu/mTNFR2-k/i mice after injection 2 days or 4 days. n = 7 mice/group, * $p < 0.05$, ** $p < 0.01$ were examined by one-way ANOVA with post-hoc comparisons of Tukey tests. Data are presented as means ± SEM.

The sensitivity of cholinergic neurons in the NBM to NMDA excitotoxicity can be quantified by the reduction of their cortical projections (Harkany et al., 2000). We therefore assessed the cholinergic fibres in the parietal cortex of all tested group in hu/mTNFR2-k/i mice by histopathological analyses. We found that NMDA-injected into the NBM significantly induced a reduction of cholinergic innervations in the layer V of the parietal cortex (Figure 4.2 and 4.3, C and G, $p < 0.0001$). However, the application of TNC-scTNF$_{R2}$ or EHD2-scTNF$_{R2}$ significantly prevented the cholinergic denervation in comparison to NMDA-injected group (Figure 4.2C and Figure 4.3C, $p < 0.0001$). Application of TNC-scTNF$_{R2}$ alone did not alter cholinergic innervations.

Furthermore, the neuroprotective effects of TNC-scTNF$_{R2}$ or EHD2-scTNF$_{R2}$ were reflected in the degree of microglial activation (Figure 4.2 and 4.3, E to H). An injection with NMDA caused a massive activation of microglia at the infused site in the NBM (Figure 4.2F), comparing to that in PBS group ($p < 0.0001$). Application of TNC-scTNF$_{R2}$ or EHD2-scTNF$_{R2}$ together with NMDA showed a significant decline of size of microglial activation in comparison to NMDA-injected group (Figure 4.2 and 4.3, G and H, $p < 0.0001$). Application of TNC-scTNF$_{R2}$ or EHD2-scTNF$_{R2}$ alone did not affect the output of microglial activation.

Taken together, these results show that TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$ remarkably prevents NMDA-induced NBM lesion in hu/mTNFR2-k/i mice.

4.3.2 ATROSAB Prevents NMDA-induced NBM Lesion in hu/mTNFR1-k/i Mice

Roles of TNFR1 signalling have been directly or indirectly verified to aggravate neurodegeneration (Fontaine et al., 2002; Williams et al., 2014). Here, we tested the potential effects of human TNFR1 antagonist ATROSAB *in vivo* in the NBM-lesioned hu/mTNFR1-k/i mice. By stereotactic injection of NMDA lesions in the NBM were generated. We found that lesioning of the NBM resulted in a decrease of body weight (Figure 4.4, $p < 0.01$) in comparison to that in control groups. However, ATROSAB showed a strong protective effect against a reduction of body weight (one-way ANOVA, $p < 0.05$).

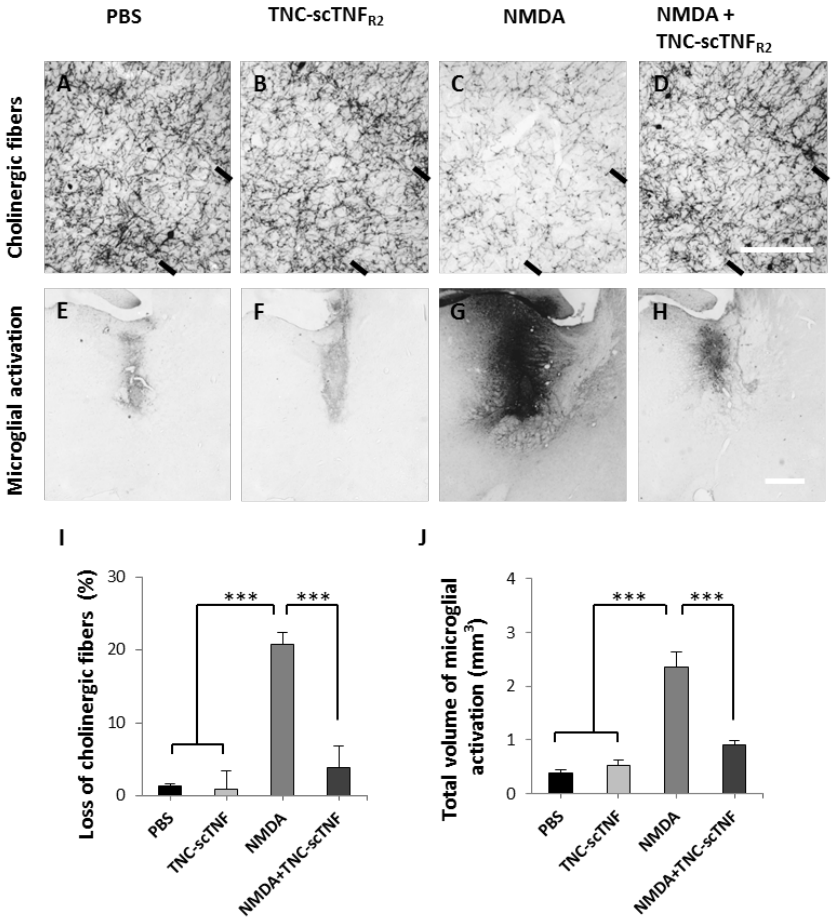

Figure 4.2. TNC-scTNF$_{R2}$ protects against NMDA-induced NBM lesion in hu/mTNFR2-k/i mice. (**A-D**) Representative images show ChAT-positive cholinergic innervations in the parietal cortex. NMDA injected into the NBM induced an extensive cholinergic fibre loss in the layer V of the parietal cortex (C), compared to the control groups (A and B). However, TNC-scTNF$_{R2}$ treatment prevented fibre loss (D). Parallel bars (A-D) indicate the layer V of the parietal cortex in which quantitative measurements were performed. (**I**) Quantification of cholinergic fibre density in layer V of the parietal cortex. Fibre density was measured in 8 sections/mouse, n = 7 mice/group. All data in bar

charts represent means ± SEM. *** p < 0.0001, one-way ANOVA with post-hoc comparisons Tukey. (**E-H**) Representative images show CD11b-positive activated microglia in the NBM. NMDA injected into the NBM induced a massive volume of microglial activation (G), compared to those in both control groups (E, F). However, TNC-scTNF$_{R2}$ treatment significantly reduced microglial activation induced by NMDA (H). (**J**) Quantification of total extent of activated microglia around the injections. Microglial activation was measured in a series of sections with microglial activation, n = 7 mice/ group. All data in bar charts represent means ± SEM. *** p < 0.0001, one-way ANOVA with post-hoc comparisons Tukey.

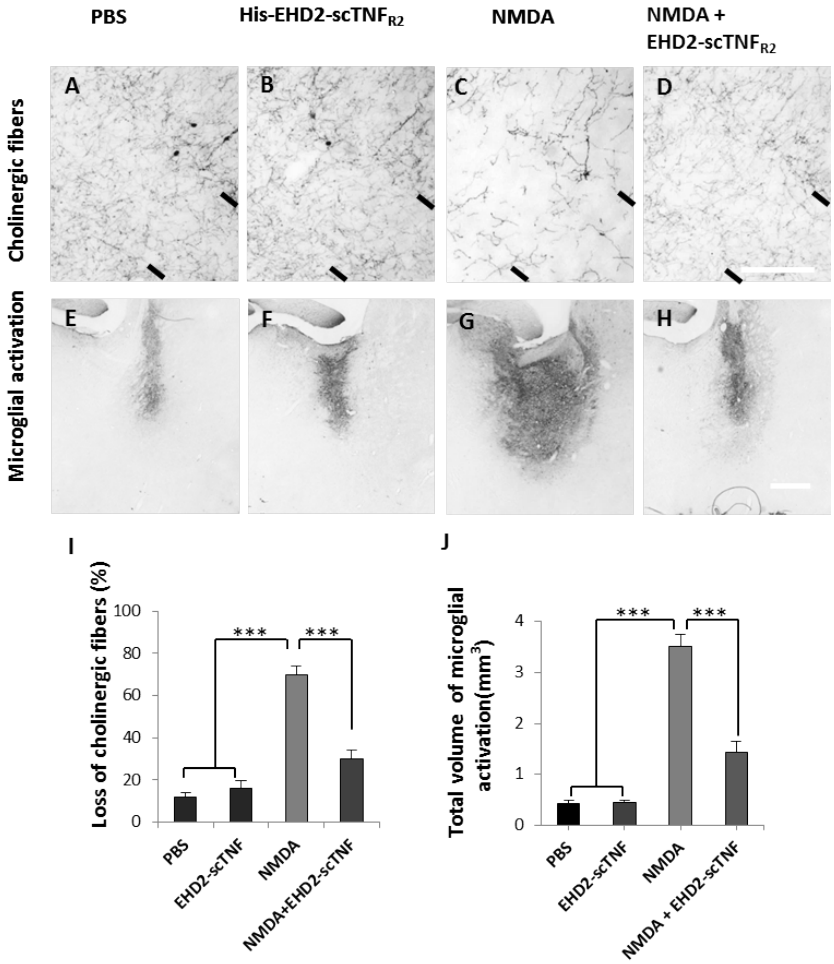

Figure 4.3. EHD2-scTNF$_{R2}$ prevents the NMDA-induced NBM lesion in hu/mTNFR2-k/i mice. (**A-D**) Representative images show ChAT-positive cholinergic innervations in the parietal cortex. NMDA injected into the NBM induced an extensive cholinergic fibre loss in the layer V of somatosensory cortex (C), compared to the control group (B). However, EHD2-scTNF$_{R2}$ treatment prevented fibre

loss (D). Parallel bars (A-D) indicate the layer V of the somatosensory cortex in which quantitative measurements were performed. (**I**) Quantification of cholinergic fibre density in layer V of the parietal cortex. Fibre density was measured in eight sections/mouse, n = 7 mice/group. All data in bar charts represent means ± SEM. *** $p < 0.0001$, one-way ANOVA with post-hoc comparisons Tukey. (**E-H**) Representative images show CD11b-positive activated microglia in the NBM. NMDA injected into the NBM induced a massive volume of microglial activation (G), compared to those in both control groups (E, F). However, EHD2-scTNF$_{R2}$ treatment significantly reduced microglial activation induced by NMDA (H). (**J**) Quantification of total extent of activated microglia around the injections. Microglial activation was measured in a series of sections with microglial activation, n = 7 mice/ group. All data in bar charts represent means ± SEM. *** $p < 0.0001$, one-way ANOVA with post-hoc comparisons Tukey.

By histopathological examination of brains from treated mice, we observed that ATROSAB administration protected against the reduction of cholinergic fibres in the parietal cortex and the massive volume of microglial activation at the site of the NBM induced by NMDA excitotoxicity (Figure 4.5 D and I, $p < 0.0001$). Injection of a control IgG (anti-huEGFR), however, did not significantly alter NMDA mediated neurodegeneration (Figure 4.5 E and J). Taken together, these data show a neuroprotective activity of ATROSAB *in vivo* that supports a previous *in vitro* study (Richter et al., 2013).

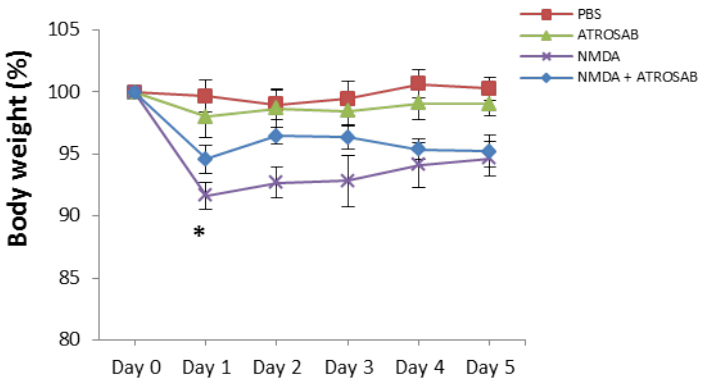

Figure 4.4. Body weight changes after pharmacological treatment in hu/mTNFR1-k/i mice. ATROSAB did prevent the significant reduction of body weight induced by NMDA injection into the NBM**,** but it only showed a statistically significant prevention on Day 1.

4.3.3 TNFR2 Signalling is Essential for ATROSAB Neuroprotection

TNF-α/TNFRs signalling pathways are complicated in neurodegenerative processes: TNF/TNFR1 signalling pathway has been reported depravation, whereas TNF-α/TNFR2 signalling pathway is neuroprotective via PKB/Akt activated NF-κB signalling pathway (Fontaine et al., 2002). Our previous studies have shown that activation of TNFR2 and inhibition of TNFR1 survived cholinergic neurons against NMDA-mediated NBM lesion (Figure 4.3, I and J). To clarify the importance of inhibition of TNFR1 and activation of

TNFR2 *in vivo*, we blocked TNFR2 signalling pathway by a mouse TNFR2 antagonist (MAB426) and meanwhile inhibited huTNFR1 signalling pathway by ATROSAB in this lesion model as shown by (Figure 4.6). We found that MAB426 completely abolished ATROSAB mediated protection on both cholinergic innervation and microglial activation (Figure 4.6 D and I).

PKB/Akt is considered to be a prominent and ubiquitous downstream target protein of PI3K (Cantley, 2002). Previously we have shown that the PI3K-PKB/Akt pathway mediates TNFR2-promoted protection from excitotoxic cell death (Dolga et al., 2008a; Fontaine et al., 2002; Marchetti et al., 2004). To further study the molecular pathways underlying the neuroprotective effects of ATROSAB, we isolated primary neurons from hu/mTNFR1-k/i mice and investigated TNF-induced phosphorylation of PKB/Akt (Ser473). As expected stimulation with a non-receptor selective mouse tmTNF-mimetic, (EHD2-sc-mTNF) induced phosphorylation of PKB/Akt. Interestingly, phosphorylation was enhanced in presence of the TNFR1 antagonist ATROSAB (Figure 4.7A), suggesting that PKB/Akt activation occurs via TNFR2 and concomitant TNFR1 signalling interferes with this pathway. In addition, TNF was shown to protect primary neurons from glutamate-induced cell death in a TNFR2-dependent manner (Marchetti et al., 2004). Similarly, we could show that the mouse TNFR2-specific TNF, EHD2-sc-mTNF$_{R2}$, protects primary hu/mTNFR1-transgenic neurons from excitotoxic cell death (Figure 4.7B). In accordance with ATROSAB mediated increase of PKB/Akt phosphorylation, ATROSAB enhanced the neuroprotective effect of EHD-sc-mTNF at lower concentrations (10 ng/ml, Figure 4.7B) in this *in vitro* cell model, too.

4.4 DISCUSSIONS

TNF-α has been reported to exert opposite effects via its two receptors, TNFR1 and TNFR2, particularly in the central nervous system(CNS) (Dong et al., 2015; Probert, 2015), suggesting that TNFR1 mediates deterioration and TNFR2 mediates protection. Investigations in a mouse model of retinal ischemia revealed that TNFR1-deficient animals were protected from ischemic lesions, whereas TNFR2$^{-/-}$ mice showed enhanced neuronal loss and a more severe pathology compared to wildtype animals (Fontaine et al., 2002). Similar, using the cuprizone-induced mouse model of demyelination, it was shown that TNFR2 is critical for oligodendrocyte regeneration, whereas TNF-α signalling via TNFR1 promoted nerve demyelination (Arnett et al., 2001). TNF-α exacerbates Aβ-induced toxicity in neurons through inhibiting TNFR2 signalling (Shen et al., 1997). These studies demonstrate that TNF-α/TNFR2 signalling pathway is neuroprotective in a neurodegenerative instance, which is opposite to the role of TNFR1. The same principle applied to oligodendrocyte progenitor cells, TNFR2 was shown to protect against oxidative stress (Maier et al., 2013). Furthermore, Fischer et al. (2011) demonstrated the generation of a human TNFR2 selective agonist (TNC-scTNF$_{R2}$) and described *in vitro* that TNC-scTNF$_{R2}$ rescues neuronal survival from oxidative stress-induced toxicity. We here showed in an *in vivo* model of NMDA induced cellular degeneration and loss of neuronal functions that

both TNFR2 selective agonists (TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$) and a TNFR1 selective antagonistic antibody (ATROSAB) exert clearly neuroprotective activity, which is in line with previous studies (Fischer et al., 2011; McCann et al., 2014).

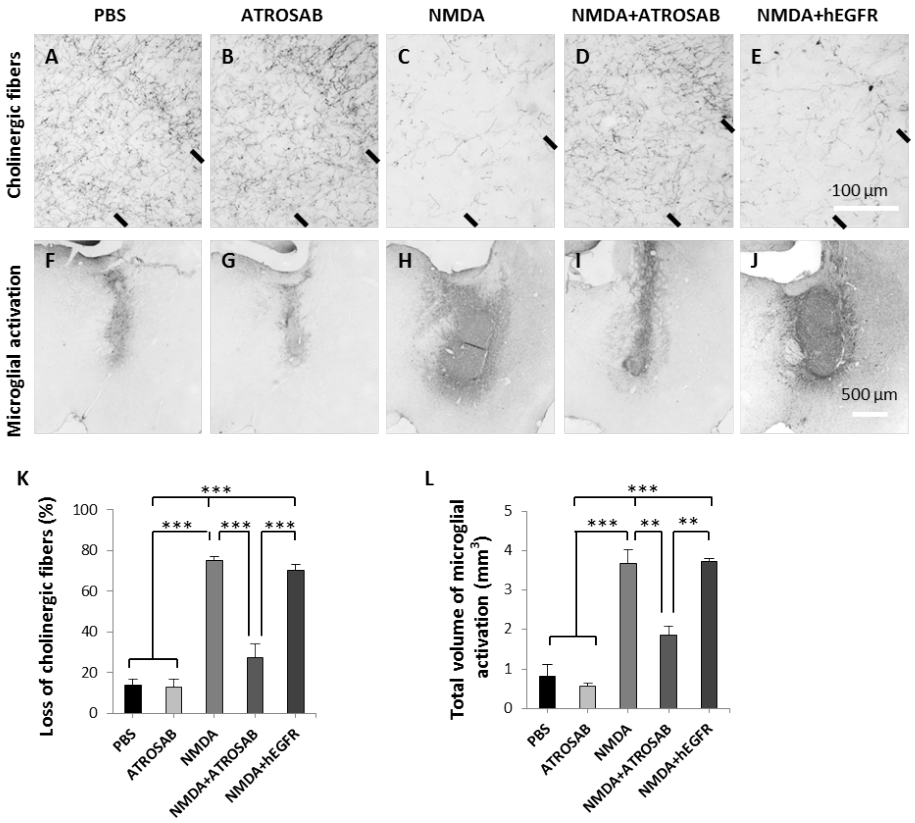

Figure 4.5. ATROSAB prevents NMDA-induced NBM lesion in hu/mTNFR1-k/i mice. (**A-E**) Representative images show ChAT-positive cholinergic innervations in the parietal cortex. NMDA injected into the NBM induced an extensive cholinergic fibre loss in the layer V of the parietal cortex (C), compared to the control group (A, B). However, ATROSAB treatment prevented fibre loss (D). A human anti-EGFR antibody (hEGFR) did not alter NMDA-induced NBM lesion. Parallel bars (A-E) indicate the layer V of the somatosensory cortex in which quantitative measurements were performed. (**K**) Quantification of cholinergic fibre density in layer V of the parietal cortex. Fibre density was measured in eight sections/mouse, n = 7 mice/group. All data in bar charts represent means ± SEM. *** $p < 0.0001$, one-way ANOVA with post-hoc comparisons Tukey. (**F-J**) Representative images show CD11b-positive activated microglia in the NBM. NMDA injected into the NBM induced a massive volume of microglial activation (H), compared to those in both control groups (F, G). However, ATROSAB administration significantly reduced microglial activation induced by NMDA (I). (J) A control IgG (anti-hEGFR) did not alter the NMDA-induced microglial activation. (**L**)

Quantification of total extent of activated microglia around the injections. Microglial activation was measured in a series of sections with microglial activation, n = 7 mice/ group. All data in bar charts represent means ± SEM. ** $p < 0.01$, *** $p < 0.0001$, one-way ANOVA with post-hoc comparisons Tukey.

Studies on lovastatin actions further support TNFR2 involvement in neuroprotection. Lovastatin is a cholesterol-lowering drug with reported neuroprotective properties that can reduce the incidence of stroke and progression of Alzheimer's disease (Elkind et al., 2008; Fonseca et al., 2009). Lovastatin increased the expression of TNFR2 in cortical neurons *in vitro* (Dolga et al., 2008b) and was neuroprotective in $TNFR1^{-/-}$neurons, whereas lovastatin's protection was lost in neurons from $TNFR2^{-/-}$ mice (Dolga et al., 2008b). Furthermore, lovastatin-mediated neuroprotection led to an increase in PI3K dependent PKB/Akt phosphorylation, whereas inhibition of PKB/Akt activation entirely abolished lovastatin-mediated neuroprotection. This is in line with previous findings that TNFR2 mediated neuroprotection is dependent on the PI3K-PKB/Akt pathway (Marchetti et al., 2004) and suggests that Lovastatin induced neuroprotection is dependent on TNFR2 signalling pathway.

In the present study, we evaluated the therapeutic potential of TNFR2 agonists TNC-$scTNF_{R2}$ and EHD2-$scTNF_{R2}$ and a TNFR1 antagonist ATROSAB in the mouse model of NMDA induced NBM lesion. Similar to lovastatin, both TNC-$scTNF_{R2}$ and EHD2-$scTNF_{R2}$ protected cholinergic neurons and neocortical innervations against NMDA induced excitotoxic damage and remarkably reduced microglial activation at the site of the lesion. In addition, ATROSAB has also shown a dominant neuroprotection against the NBM damage. ATROSAB mediated neuroprotection from neurodegeneration was found to be linked to an enhancement of TNFR2 signalling leading to PKB/Akt activation. This is evident from abrogation of neuroprotection *in vivo* upon co-treatment with TNFR2 blockers and from *in vitro* studies showing increased phosphorylation of PKB/Akt as well as enhanced resistance of primary cortical neurons towards excitotoxic insult by the TNFR2 selective TNF in the presence of TNFR1 blockade. The PKB/Akt pathway is known to be essential for several physiological processes, including cell differentiation, regeneration and cell survival (Skaletz-Rorowski et al., 2003). TNFR2 promotes PKB/Akt activation and translocation via phosphorylation at Ser473 in neuronal cells (Marchetti et al., 2004). Our data showed that in cortical neurons EHD2-sc-$mTNF_{R2}$ was able to activate PKB/Akt (Ser473), and this activation was further increased significantly by ATROSAB. Together these data *in vivo* and *in vitro* suggest that TNFR2 -PKB/Akt signalling pathway is essential in ATROSAB-mediated neuroprotection.

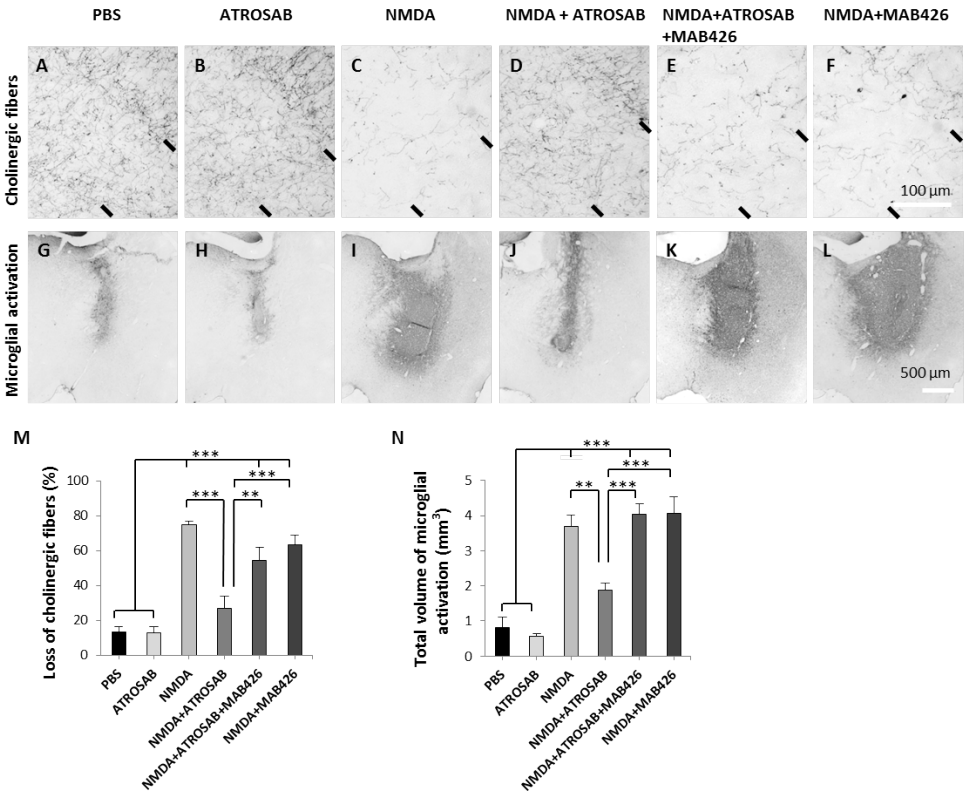

Figure 4.6. ATROSAB prevents NMDA-induced NBM lesion in hu/mTNFR1-k/i mice mainly via indirect activation of TNFR2. (A-F) Representative images show ChAT-positive cholinergic innervations in the parietal cortex. NMDA injected into the NBM induced an extensive cholinergic fibre loss in the layer V of the parietal cortex (C), compared to the control groups (A and B). However, ATROSAB treatment attenuated fibre loss (D). ATROSAB mediated protection against fibre loss was prevented by TNFR2 antagonistic MAB426 (E). However, MAB426 alone did not significantly alter NMDA-induced NBM lesion (F). Parallel bars indicated the layer V of the somatosensory cortex in which quantitative measurements were performed. Scale bar is 100 µm in (F). (**M**) Quantification of cholinergic fibre density in layer V of the somatosensory cortex. Fibre density was measured in 8 sections/mouse, n = 7 mice/group. All data in bar charts represent means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, one-way ANOVA with post-hoc comparisons Tukey. (**G-L**) Representative images show CD11b-positive activated microglial in magnocellular nucleus basalis. NMDA injected into the NBM induced a massive volume of microglial activation (I), compared to those in the groups (G and H). However, ATROSAB treatment significantly reduced microglial activation induced by NMDA (J). ATROSAB neuroprotection against microglial activation was prevented by TNFR2 antagonistic MAB426 (K). MAB426 did not significantly alter NMDA-induced NBM lesion (L). Scale bar is represent 500 µm in (L). (**N**) Quantification of total extent of activated microglial infiltration around the injections. Microglial activation was measured in a series of sections with microglial

activation, n = 7 mice/ group. All data in bar charts represent means ± SEM. ** p < 0.01, *** p < 0.0001, one-way ANOVA with post-hoc comparisons Tukey.

Here, we provide compelling evidence NMDA induced acute neuronal lesions that abrogation of complete TNF signalling by blocking TNFR1 by ATROSAB is neuroprotective. We propose that the neuroprotective activity of ATROSAB is accomplished by a twofold action: As a consequence of the excitotoxic insult activated microglia promotes, via soluble TNF production, the expansion of the degenerative tissue response in an autocrine TNFR1 dependent way; inhibition of TNFR1 limits this process. At the level of neuronal cells, it was shown previously that proper TNFR2 activation induces protection from excitotoxicity *in vitro* (Dolga et al., 2008b; Marchetti et al., 2004); in the *in vivo* model analyzed here, in neurons in the vicinity of the acute insult and not immediately succumbing to excitotoxic death, a competition of TNFRs for limiting amounts of endogenously produced membrane form of TNF molecules occurs, which results in suboptimal activation of TNFR2 of this cell population under a non-treatment condition. Moreover, at the level of intracellular signalling, it is known that signal crosstalk between TNFR1 and TNFR2 exists in terms of competition for common signal transducers like TRAF2 (Fotin-Mleczek et al., 2002; Weiss et al., 1997), with TNFR1 outperforming TNFR2 pathways in the case of abundance of soluble TNF-α, which triggers exclusively TNFR1 (Probert, 2015). In the presence of ATROSAB, neuronal response is shifted towards TNFR2 because of blocked neuronal TNFR1, allowing the induction of a resistant state by endogenous mTNF-α.

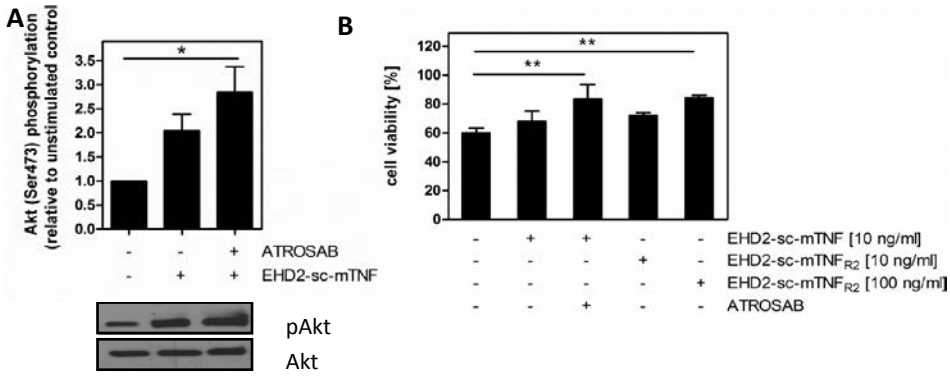

Figure 4.7. ATROSAB is neuroprotective via enhanced TNFR2 signalling. (**A**) Primary neurons, isolated from hu/mTNFR1-k/i mice, were stimulated with or without ATROSAB (100 µg/ml) for 30 min followed by addition of Blot (n = 4, ± SEM). (**B**) Primary neurons, isolated from hu/mTNFR1-k/i mice, were stimulated wildtype EHD2-sc-mTNF (10 ng/ml). Then cells were incubated for 24 h, lyzed and phosphorylation of Akt was quantified by Western with or without ATROSAB (100 µg/ml) for 30 min followed by addition of wildtype EHD2-sc-mTNF or mouse TNFR2-selective EHD2-sc-mTNF$_{R2}$. After 24 h, glutamate (5 µM) was added and cells were incubated for an additional hour. Then medium was exchanged to remove glutamate and cells were incubated for 23 h. Cell viability was

determined by MTT assay (n = 3). All data in bar charts represent means ± SEM. ** $p < 0.01$, one-way ANOVA with post-hoc comparisons Tukey.

Glutamate, a major neurotransmitter, can interact with the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors. Exacerbated activation of these glutamate receptors, specifically the increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) induces disturbances in the $[Ca^{2+}]_i$ homeostasis, which finally leads to progressive neuronal cell death and is a hallmark of acute and chronic neurological diseases (Harkany et al., 2000; Mattson, 2004; Donnelly and Popovich, 2008). It seems that targeting NMDA receptors by inhibition might be protective against neurotoxicity. In contrast, a number of articles have shown that NMDA receptor antagonists such as dizocilpine (MK-801) lead to neuronal apoptosis by triggering caspase-3 activation (Inta et al., 2015), and impair memory functions in mice and in rats (Smith et al., 2016; Tomazi et al., 2016). Thus, NMDA receptor antagonists as therapeutic strategy against excitotoxicity are not valid. However, TNF, a pro-inflammatory factor, induces cell apoptosis by prevention of calcium release (Yan et al., 2015). Interestingly, activation of potassium intermediate/small conductance calcium-activated channel $K_{Ca}2$ prevented $[Ca^{2+}]_i$ deregulation and reduced neuronal death following glutamate toxicity and cerebral ischemia (Dolga et al., 2011), proposing that $K_{Ca}2$ channels activation might be a potential treatment strategy to alleviate damage in the course of acute or chronic neurodegenerative disorders. It was previously demonstrated that a potential molecular mechanism of TNFR2 mediated protection against glutamate excitotoxicity was associated with an increased expression of $K_{Ca}2.2$ channel (Dolga et al., 2008a) outlining a potential molecular mechanism of TNFR2-mediated protection of neurons from death during exposure of a priori excitotoxic stimuli. Since TNFR2 signalling protects neurons from glutamate induced excitatory cell death *in vitro*, ligands promoting TNFR2 signalling might be superior to NMDA antagonist as they do not completely inhibit glutamate induced signal transmission, but buffer excitotoxicity likely by acquisition of a resistant state of affected cells. Because of the antithetic action of TNF via its two receptors, we reasoned that a selective activation of TNFR2 signalling could shift the balance of endogenous TNF activity towards an overall neuroprotective/regenerative response. Additionally, TNFR2 selective agonists seem to be particularly suitable to treat inflammatory, demyelinating diseases, because next to the direct neuroprotective effects shown in this report, data from different laboratories outline that TNFR2 is also involved in immune suppression via expansion and stabilization of regulatory T cells (Chen et al., 2007, 2008, 2010a, 2010b, 2013; Okubo et al., 2013) and induces remyelination (Arnett et al., 2001; Fischer et al., 2014; Patel et al., 2012). Thus, TNFR2 agonists might promote therapeutic effects via multiple cellular targets.

Importantly, initial studies with anti-TNF-α therapeutics showed that TNF blocking drugs cannot be used to treat neurodegenerative diseases, such as MS. In support of this, we here provide compelling evidence in an *in vivo* model of NMDA induced acute neuronal lesions that abrogation of complete TNF signalling by blocking both TNFRs is not protective, because mounting of a neuroprotective TNFR2 dependent response is prevented. However, pre-clinical studies in the EAE model (Brambilla et al., 2011;

Evangelidou et al., 2014; Taoufik et al., 2011) or a mouse model of spinal cord injury (Novrup et al., 2014) revealed that selective neutralization of sTNF/TNFR1 signalling is neuroprotective. Of note, in the EAE model, systemic application of TNFR1 blocking antibodies proved to be therapeutically effective (Williams et al., 2014). Accordingly, TNFR1 antagonists such as ATROSAB should be superior to conventional anti-TNF drugs in the treatment of neurodegenerative diseases, as they spare TNFR2 but still block detrimental signals transmitted via TNFR1.

In summary, this translational study reveals that activation of TNFR2 through using TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$ indeed protects against neurodegenerative physiopathology *in vivo* in hu/mTNFR-k/i mice. Furthermore, these drugs did not take any adverse effects for animals during the experimental processing. These suggest that TNC-scTNF$_{R2}$ and EHD2-scTNF$_{R2}$ are worth to be considered to develop the therapeutic agents in AD. Apparently, we provide proof that these drugs could be further developed for the treatment for the clinical trials with AD. Our study has been focused on mice with acute pathological processes (the NBM lesion model). Before translation of this strategy into human patients with AD, further development of this approach is essential. For instance, firstly these pharmacological drugs should be assessed the potent against the cognitive deficits induced by the NBM lesion, and then evaluated their therapeutic potent against the chronic pathophysiology in AD transgenic mice. It is worthwhile in addition to test the capability of these compounds for crossing the blood brain barrier or to modify these compounds accordingly. Although we did not find any adverse effects of these drugs in our experimental trials, further information on potential side effects and pharmacokinetics are needed.

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Chapter 5

Blocking TNFR1 and Activating TNFR2 Reverses NBM Lesion Mediated Cognitive Dysfunctions

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ABSTRACT

The nucleus basalis Meynert (NBM) is a main source of cholinergic innervations to cognitive areas of the brain. Cholinergic denervation in the cerebral cortex induced by the NBM lesion causes memory dysfunctions that resemble cognitive deficits in AD patients. Previous studies have shown *in vitro* and *in vivo* that NMDA-mediated excitotoxicity can be prevented by TNF receptor 2 (TNFR2) signalling pathway whereas exacerbated by TNF receptor 1 (TNFR1) signalling pathway. This suggests that selective targeting TNFRs via activation of TNFR2 and/or inhibition of TNFR1 could be effective against neurodegeneration. In the present study, we established the NBM lesion model which was induced by NMDA excitotoxicity, tested cholinergic system-related cognitive functions and evaluated the therapeutic effects of a selective TNFR2 agonist EHD2-scTNF$_{R2}$ and a specific TNFR1 antagonist ATROSAB. We found that the NBM lesion led to the cortical functional impairment by showing performance in a passive avoidance paradigm but did not affect hippocampus- or amygdala-related behavioral functions. Of note, the cortical functional impairment was reversed by EHD2-scTNF$_{R2}$ and ATROSAB treatments. Therefore, selective TNFR targets could possibly be a promising therapeutic strategy for AD drugs in future.

5.1 INTRODUCTION

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is considered to be the most common cause of dementia. In addition to pathological features of extracellular amyloid β (Aβ) plaques and intracellular neurofibrillary tangles (NFTs) (Flament and Delacourte, 1989; Smith and Perry, 1997), depletion of acetylcholine (Ach) and cholinergic neurons and cholinergic innervations in basal forebrain has been considered as a reliable pathological hallmark of AD (Davies and Maloney, 1976; Norvin et al., 2015). Cholinergic innervations in the cerebral cortex are reduced significantly in early onset of AD patients (Whitehouse et al., 1982), which are mainly projected by cholinergic neurons in the nucleus basalis of Meynert (named nucleus basalis magnocellularis in mice, NBM) (Whitehouse et al., 1981). In AD patients cholinergic neurons in the NBM are also reduced, a loss ranging from 44% to 76% (Tagliavini and Pilleri, 1983). Coyle et al. (1983) reported that degeneration of acetylcholine-releasing neurons and neocortical cholinergic innervations play a dominant role in memory dysfunctions such as learning performance and memory formation deficits which mimics cognitive dysfunction in AD patients. Also, Iraizoz et al. (1999) showed that damage of cholinergic neurons localized in NBM and their cortical projections correlated with the deficits of memory and learning functions in AD. Consistent with this model, studies have shown that disrupted cholinergic projections to the cerebral cortex by NMDA-mediated NBM lesion indeed leads to memory deficits (Dolga et al., 2009). These data therefore suggest that the cortical cholinergic denervation induced by NBM lesion in animals is a well-established lesion model that can be used to investigate the potential of therapeutics by showing cholinergic innervations and cognitive functions.

Tumor necrosis factor alpha (TNF-α), a major pro-inflammatory cytokine, has been implicated an involvement in the pathology of neurodegenerative diseases, such as AD and multiple sclerosis (MS) (McAlpine et al., 2009; Tan et al., 1999; Tarkowski et al., 2000; Yang et al., 2013). It has been reported that TNF-α through its receptor one (TNFR1) increases N-methyl-D-aspartate (NMDA)-mediated neuronal excitotoxicity but survives neurons via its receptor two (TNFR2) against excitotoxicity of NMDA (Fontaine et al., 2002), indicating that inhibition of TNFR1 or/and activation of TNFR2 might be as a novel and potential therapeutic strategy against neurodegeneration. Recently, a few studies have just started to report potential therapeutic effects via inhibition of TNFR1 or activation of TNFR2 pathway. For instance, a recombinant human TNFR1 selective antagonist prevents the symptoms of EAE mouse model (Williams et al., 2014); also, a humanized TNFR2 specific agonist showed to rescue neurons against oxidative stress (Fischer et al., 2011).

The objective of the present study was to investigate the functional roles in cognitive processing affected by the damage of cholinergic system, and to assess the therapeutic effects of both a human TNFR2 selective agonist EHD2-scTNF$_{R2}$ and a human TNFR1 specific antagonist ATROSAB in humanized TNFR knock-in (hu/mTNFR1-k/i and hu/mTNFR1-k/i) mice. We here focused on the NBM projected cholinergic innervations-related cognitive functions because it appears to be significantly reflected in AD. Through

testing mouse cognitive functions that relate with the NBM cholinergic projections including the short-term memory, animal anxiety and long-term memory, in a spontaneous alternation task, an elevated plus maze and a passive avoidance paradigm, we found that NMDA-induced NBM lesion-mediated cholinergic denervation did not affect the short-term memory and animal anxiety while devastated the long-term memory; however, both EHD2-scTNF$_{R2}$ and ATROSAB reversed the long-term memory impairments.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Male homozygous hu/mTNFR-k/i C57BL/6J mice (12 weeks, 24-30 g) were established by using genetic techniques. All animals were individually housed with a free access to *ad libitum* food and tap water and kept in an air-conditioned room (21 ± 2°C) with a 12/12 h light-dark circle (lights on 7:00 a.m.). Animal care and treatment were carried out in accordance with the local Ethical Committee guidelines on the use of experimental animals at the University of Groningen, the Netherlands (DEC6523) and University of Stuttgart, Germany (35-9815.81-0350).

5.2.2 Nucleus Basalis Injection

An injection into the nucleus basalis magnocellularis (NBM) was performed as previously described by Luiten et al. (1995). After anaesthesia with isoflurane, mouse heads were mounted in a Kopf stereotactic frame (Kopf Instruments model 900, Tujunga, CA, USA). A volume of 0.6-µl solution was slowly infused in two steps of 0.3 µl into the NBM of the right hemisphere (coordinates: 0.6 mm posterior to bregma; 2.1 mm lateral to the sagittal suture; 4.6/4.4 mm ventral to the dura.) (Paxinos and Franklin, 2001). The contralateral intact hemisphere served as internal control without interference. After each slow injection (0.1 µl/min), the needle of a 1-µl Hamilton syringe was left in situ for 2 min for effective drug diffusion.

5.2.3 Behavioral Evaluation

All hu/mTNFR-k/i mice were performed a unilateral injection at the right site of NBM. Hu/mTNFR1-k/i mice were divided into four groups: (1) phosphate-buffered saline (PBS, 0.01 M, pH = 7.40); (2) 3 µg ATROSAB (descripted by Zettlitz et al., 2010); (3) 55 nmol NMDA (Sigma, USA); (4) 55 nmol NMDA together with 3 µg ATROSAB. Hu/mTNFR2-k/i mice were also divided into four groups: (1) PBS; (2) 580 ng His-EHD2-scTNF$_{R2}$ descripted by (Grell et al., 1995); (3) 55 nmol NMDA; (4) 55 nmol NMDA together with 580 ng EHD2-scTNF$_{R2}$. After 5 days, memory performances were assessed as shown by the time outline (Figure 5.1).

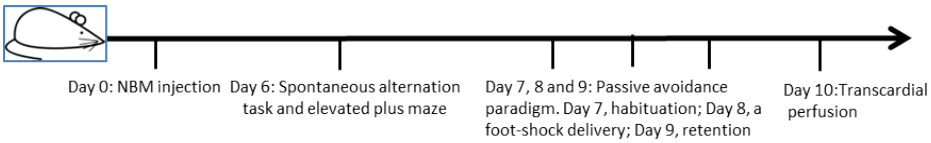

Figure 5.1. Schematic outline of the experimental setup. hu/mTNFR-k/i mice received a unilateral infusion into the NBM. Five days upon the injection, the cognitive performances of all mice were tested in a spontaneous alternation task, an elevated plus maze and a passive avoidance paradigm to evaluate their short-term memory, animal anxiety and long-term memory.

5.2.4 Spontaneous Alternation Task

Evaluation of spontaneous alternation behavior in a Y-maze to investigate spatial short-term memory (working-memory) was performed as described previously (Dolga et al., 2009). Briefly, the device consisted of three transparent Plexiglas and tubular arms (5 cm in diameter, 27.5 cm length) with an angle of 120°. Mice were placed in the maze center and allowed to freely explore for 8 min. The sequence of arm entries was recorded visually. A criterion was considered that a mouse complete entered one arm with all four paws. A successful spontaneous alternation was defined as entries into all three different arms by consecutive choices. The percentage of spontaneous alternation was calculated as the ratio of actual to the maximum possible number of alternations. The maximum possible number of alternations was defined as the total number of arm entries minus two.

5.2.5 Elevated Plus Maze

Anxiety-related behaviors performed in an elevated plus maze were as described previously (Harkany et al., 2000). The plus maze consisted of two open arms (5.5 cm width x 30 cm length) and two enclosed arms (5.5 cm width x 30 cm length x 15 cm high) and was elevated 50 cm above the ground. After 5 days upon the NBM injection, mice were placed the center of the maze (5.5 cm x 5.5 cm) and allowed to explore freely for 8 min. A criterion was considered that a mouse complete entered one arm with all four paws. The time spent on the open or closed arms, the number of arm entries and total entries were recorded. The correlation values of this apparatus were calculated as a percentage time spent on open arms, a percentage time spent on closed arms or a percentage of time spent on center area, a percentage of number of open arm entries (the time spent on open arms/ total time x 100%, the time spent on closed arms/ total time x 100%, the number of entering open arms/ total arm entries x 100%).

5.2.6 Passive Avoidance Paradigm

A one-trial learning, step-through passive avoidance test was performed in an apparatus (Venault et al., 1986). This device was divided into two compartments by a retractable door, an illuminated compartment with a plastic floor and a dark compartment with a grid floor. The illuminated compartment was lighted by a 12-watt light bulb. After an injection

into the NBM 6 days, mice were firstly acclimatized to situations in the experimental equipment. Mice were placed in the illuminated platform facing to the closed retractable door. The retractable door was raised 60 sec later, and mice were allowed to explore freely in the apparatus for 2 min, thereafter returned to home-cages. The training tests were performed after 24 h. Mice were placed in the illuminated compartment facing to the closed door and allowed to explore for 60 sec. The door was raised, and a latency defined as the time period since the door was opened until animals entered the dark compartment with all four paws (pre-shock latency) was recorded. After mice completely entered the dark compartment, the retractable door was closed, and a foot-shock (0.3 mA, 2 sec) was delivered through the metal grid floor. After 30 sec mice were removed to home cages. Memory retention was performed after training 24 h. Each animal was placed in the illuminated compartment for 60 sec, after opening the door, a latency (post-shock latency) to enter the dark compartment (as described in the training test) was recorded up to a maximum of 8 min.

5.2.7 Statistical Analysis

Neurotoxic effects of NMDA infusion and the efficacy of compound treatments in behavioral tests were calculated by using one-way ANOVA analysis of variance with comparison of post-hoc Tukey tests. A value of $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was considered as statistical significance for the vaccine treatment. Data were presented as means ± SEM.

5.3 RESULTS

5.3.1 NMDA-induced NBM Lesion Does not Alter the Short-term Memory in Both hu/mTNFR1-k/i and hu/mTNFR2-k/i Mice

Through histopathological analyses, NMDA excitotoxicity indeed induced a significant reduction of cholinergic neurons in the NBM; however, cholinergic neuron loss was prevented by EHD2-scTNF$_{R2}$ and ATROSAB, in both hu/mTNFR2-k/i mice and hu/mTNFR1-k/i mice (unpublished data). To investigate whether the NBM lesion can lead to cognitive dysfunctions and to assess the potential therapeutic effects of EHD2-scTNF$_{R2}$ and ATROSAB, all experimental mice were performed memory tests as shown by a time outline (Figure 5.1). After five days upon an infusion into the NBM, experimental mice were tested in a spontaneous alternation task to evaluate their short-term memory (Figure 5.2). Spontaneous alternation behaviour is generally regarded as a measure of short-term memory (working memory) (Dudchenko, 2004). We found that the NMDA-induced NBM lesion and the compounds tested did not affect the short-term memory by shown the alternation rate and the frequency of entries in both mouse strains.

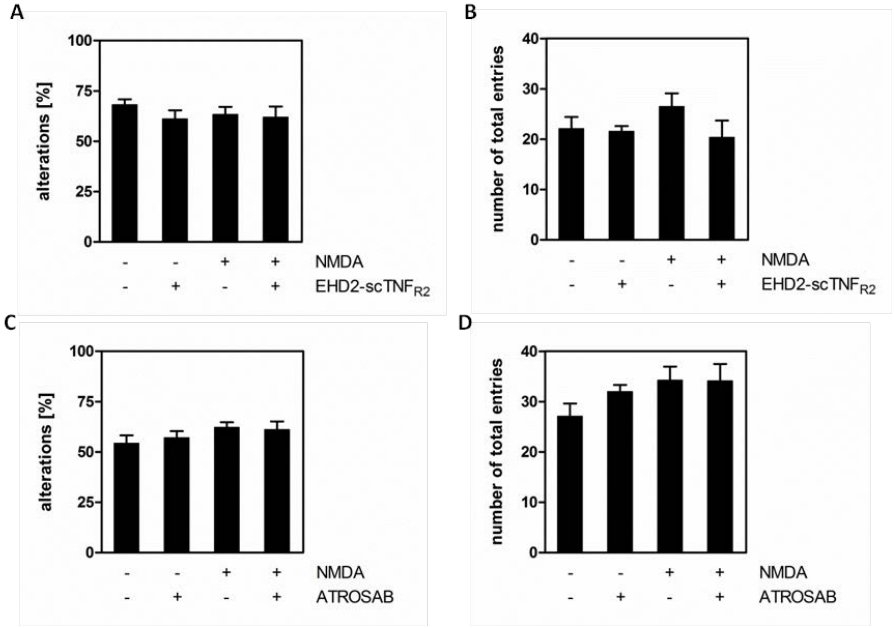

Figure 5.2. The NBM lesion does not trigger short-term memory impairment in both hu/mTNFR1-k/i and hu/mTNFR2-k/i mice. NBM injection of NMDA and/or EHD2-scTNF$_{R2}$ had no effect on short-term memory, presented as (**A**) percentage of alternations and (**B**) number of total entries which were measured with spontaneous alternation in hu/mTNFR2-k/i mice. Moreover, NBM injection of NMDA and/or ATROSAB did not influence (**C**) percentage of alternations and (**D**) number of total entries in hu/mTNFR1-k/i mice. n = 7 - 9 mice/ group, bars indicate means ± SEM. All data were examined by one-way ANOVA analysis with post-hoc comparisons Tukey.

5.3.2 NMDA-induced NBM Lesion Does not Influence Animal Anxiety in Both Transgenic Mouse Lines

Subsequently, we examined experimental mice in an elevated plus maze to evaluate their anxiety-like behaviors. Our data showed no significant changes in the total number of entries (Figure 5.3 A and D), entries into the open arms (Figure 5.3 B and E) and time spent in the dark and light arms, and center of the maze (Figure 5.3 C and F) among all of the tested groups.

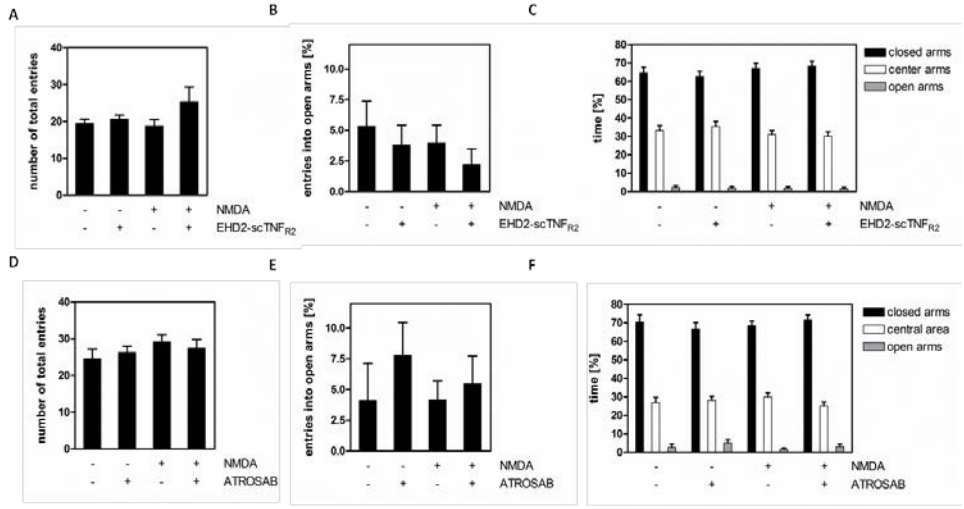

Figure 5.3. NMDA-induced NBM lesion does not cause animal anxiety in both transgenic mouse lines. Evaluation of anxiety-like behaviours by elevated plus maze in hu/mTNFR2-k/i mice showed no significant differences between the groups in the (**A**) number of entries into the different arms, (**B**) entries into the open arms and (**C**) time spent in the different arms of the maze. Moreover, in hu/mTNFR1-k/i mice there were no significant differences between groups as well on (**D**) the number of total entries, (**E**) the entries into open arms and (**F**) the time spent in the different arms of the maze. n Tukey tests.

5.3.3 Both EHD2-scTNF$_{R2}$ and ATROSAB Reverse Retention Memory Deficits Induced by the NBM Lesion

Cholinergic innervations are considered to modulate directly neocortical memory and retention functions (Pearson et al., 1983). The passive avoidance paradigm is a memory and retention task, which depends, in part, on proper neocortical memory and retention functions. Here, we used this behavioral task to evaluate whether the NBM lesion could result in neocortical memory dysfunctions and the potential effects of EHD2-scTNF$_{R2}$ and ATROSAB. Results from the passive avoidance paradigm showed that NMDA and application of EHD2-scTNF$_{R2}$ or ATROSAB did not alter the pre-shock latency to enter the dark compartment in all mice tested (Figure 5.4 A and C). However, NMDA injected into the NBM caused a significant impairment in the post-shock latency in both transgenic mouse lines (Figure 5.4 B and D). Importantly, both EHD2-scTNF$_{R2}$ and ATROSAB obliterated NMDA mediated memory impairments, respectively (Figure 5.4 B and D). EHD2-scTNF$_{R2}$ and ATROSAB alone had no effects on post-shock latency (Figure 5.4 B and D).

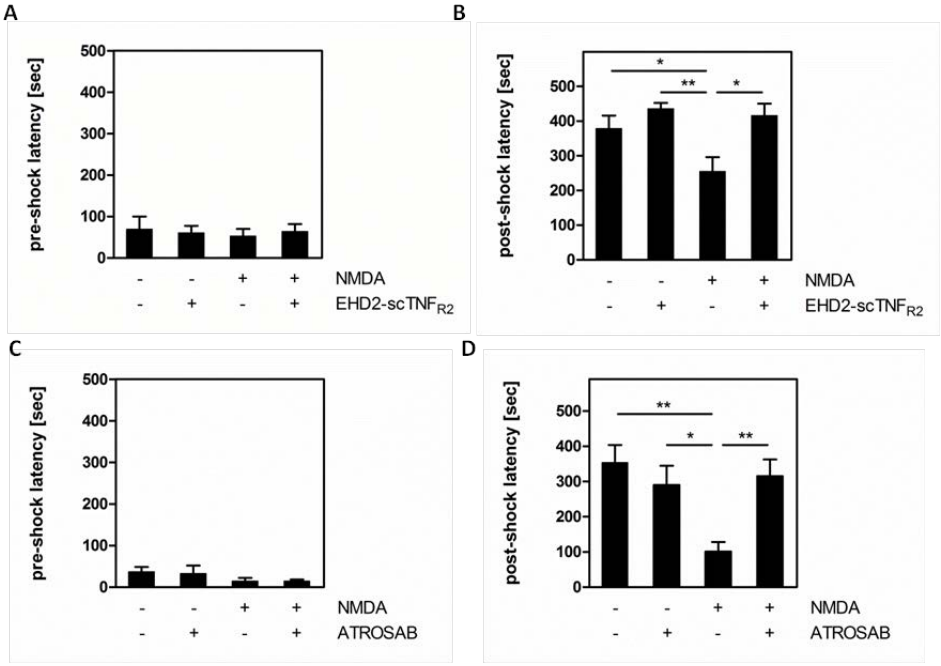

Figure 5.4. Both ATROSAB and EHD2-scTNF$_{R2}$ prevent the NBM lesion-induced memory deficits. Assessment of long term memory with a passive avoidance paradigm showed no significant differences pre-shock latency between the experimental groups of hu/mTNFR2-k/i and hu/mTNFR1-k/i mice in (**A** and **C**). NBM injection of NMDA caused a significant impairment of long term memory, measured as post-shock latency, which was obliterated by co-treatment of EHD2-scTNF$_{R2}$ in (**B**). (**D**) ATROSAB treatment preserved the post-shock latency. n = 7 - 9 mice/ group, all bars indicate means ± SEM * p <0.05, ** p < 0.01, one-way ANOVA with post-hoc comparisons Tukey.

5.4 DISCUSSION

Post-mortem studies of AD patient brains have shown neurodegenerative devastation in cholinergic areas (Wilson et al., 2009). Since cholinergic innervations to cognitive areas of the brain are mainly contributed by the nucleus basalis of Meynert (NBM), investigation of the NBM role is paid more attention. Whitehouse et al. (1981) have demonstrated that neocortical cholinergic denervation is a hallmark of early-onset of AD. Furthermore, cholinergic neurons as well as the expression of choline acetyltransferase (ChAT) in the NBM are reduced in the elderly with mild cognitive impairment and early stage of AD (Gilmor et al., 1999). These studies suggest that NMDA lesion could lead to cognitive dysfunctions. Indeed, the NBM lesion has been shown to cause memory deficits in rats (Harkany et al., 2001). Here, we established the NBM lesion model by an injection of NMDA in humanized TNFR knock-in (hu/mTNFR1-k/i and hu/mTNFR2-k/i) mice, tested

cognitive functions related with cholinergic system, and evaluated the therapeutic effects of a human TNFR2 selective agonist EHD2-scTNF$_{R2}$ and a human TNFR1 specific antagonist ATROSAB. Results of the present study showed a significant impairment on memory and retention performance in the NBM lesioned groups in both transgenic mouse strains (Figure 5.4 B and D), which is in line with previous studies in C57BL/6J mice and rats (Dolga et al., 2009; Harkany et al., 2001), while targeting TNFRs via EHD2-scTNF$_{R2}$ and ATROSAB treatments reversed the retention performance deficits, respectively. Furthermore, our data showed that the NBM lesion did not alter short-term memory and animal anxiety in both transgenic mouse models.

N-methyl-D-aspartate (NMDA) that stimulates the activation of NMDA receptors of postsynaptic neuron activates calcium channel and results in calcium overload (Que et al., 2016). Much Ca^{2+} influx leads to acute oedema in the cell, and secondary cell toxicity ultimately triggers neuron apoptosis. NMDA injected into the NBM leads to apoptosis of cholinergic neurons, and thereby affects cholinergic system in the brain. The cholinergic system projected by cholinergic neurons in the NBM can influence and regulate various tissue functions such as cerebral cortex (Dolga et al., 2009), hippocampus (Rispoli et al., 2008; Swarowsky et al., 2008), as well as amygdala (Nagai et al., 1982; Woolf and Butcher, 1982). In order to assess whether the NBM lesion by NMDA injection could lead to cognitive dysfunctions and whether EHD2-scTNF$_{R2}$ and ATROSAB could be able to counteract the cognitive deficits, all tested mice were performed in a spontaneous alternation task, an elevated plus maze and a passive avoidance paradigm. Since spontaneous alternation in a Y-maze is commonly associated with the functions of hippocampus and prefrontal-related short-term memory (Dudchenko, 2004; Senechal et al., 2008), here we used this task to evaluate the selectivity of the NBM lesion to hippocampus. Our data showed that the NBM lesion did not affect the short-term memory in transgenic mice, which is consistent with previous studies in which the NBM lesion unaffected the short-term memory of C57BL6/J mice and rats (Dolga et al., 2009; Gaykema et al., 1992), suggesting that hippocampus are not affected by NMDA-induced NBM lesion. Also, a recent study has shown that the loss of cholinergic NBM cells results in reduced cholinergic afferentiation to the neocortex rather than hippocampus (Ljubojevic et al., 2014). Furthermore, a few studies have reported mice with AD have no short-term memory deficits (Carroll et al., 2007; Zhang et al., 2010).

Moreover, to elicit whether the NBM lesion could affect amygdala-related anxiety behaviours, the NBM-lesioned and EHD2-scTNF$_{R2}$ and ATROSAB treated mice were tested in an elevated plus maze. This task is usually used to assessed the anxiety behaviours impacted by the amygdala (Pockros-Burgess et al., 2014). We observed that the NBM lesion did not alter animal anxiety-like behaviours, including the total number of entries, entries into the open arms and time spent in the dark and light arms, and center of the maze in all of the mice tested (Figure 5.3). The cholinergic innervations of human amygdala complex has been shown to be unaffected by AD whereas a significant reduction of cholinergic innervations was found in cerebral cortex (Emre et al., 1993). Our present study also showed that NMDA-mediated NBM lesion does not affect amygdala-related behaviors in hu/mTNFR2-k/i and hu/mTNFR1-k/i mice, which is in line with Emre's

research. In contrast, Harkany et al reported that NMDA-mediated NBM lesion led to rat anxiety behaviours (Harkany et al. 2000). The paradoxical results might be led to by different animal models.

Additionally, we tested mice in a passive avoidance paradigm. This device is considered to be one of the test conditions of choice for behavioural assessment of the integrity of the neocortical system (Senechal et al., 2008). We found that NMDA excitotoxicity lesioned NBM led to a memory retention deficit by records of post-shock latency in both transgenic mice (Figure 5.4 B and D), in accordance to that in C57BL/J6 mice (Dolga et al., 2008) and in rats (Harkany et al., 2001; Harkany et al., 1995). However, the retention deficit was reversed by EHD2-scTNF$_{R2}$ and ATROSAB. Our data showed that EHD2-scTNF$_{R2}$ and ATROSAB indeed can prevent NMDA-induced damage to the NBM and improved neocortical memory dysfunctions. Similar to our findings, a number of studies have shown that the NBM lesion caused memory retention deficits was reversed by Lovastatin in C56BL/6J mice and rats (Zhao et al., 2010; Dolga et al., 2009). Interestingly, the Lovastatin was shown to exert a neuroprotective effect via the activated TNFR2 signalling pathway (Dolga et al., 2008). Furthermore, our unpublished data showed that ATROSAB induced protection was dependent on TNFR2 signalling pathway. These suggest that TNFR2 is an essential role in improvements neocortical memory deficits induced by the NBM lesion.

TNF-α, a pro-inflammatory cytokine, is involved in the physiopathology in neurodegenerative disorders, such as AD (Lourenco et al., 2013). TNF-α binding to TNFR1 exerts exacerbation whereas its binding to TNFR2 is involved in neuroprotection (Fontaine et al., 2002). TNFR1 inhibition has been shown to prevent neurodegeneration and neuron cell death *in vivo* and *in vitro* (Liu et al., 2014; McAlpine et al., 2009; Williams et al., 2014). Fischer et al. (2011) illuminated that TNFR2 agonist rescues human neurons against oxidative stress. In the present study, we confirmed that functional consequences of NMDA induced-NBM lesions and the therapeutic activity of an activation of TNFR2 EHD2-scTNF$_{R2}$ and an inhibition of TNFR1 ATROSAB became phenotypically apparent in behavioural performance studies: Damage to the NBM selectively affected neocortical cholinergic denervation-related memory dysfunctions, while leaving particular hippocampal innervation and functions as well as amygdala innervations and its anxiety functions were unaffected. Treatment of such animals with either EHD2-scTNF$_{R2}$ or ATROSAB fully restored the affected cortical cholinergic memory function. Our findings increase the possibility for EHD2-scTNF$_{R2}$ and ATROSAB to apply to the treatment in AD.

In a word, the therapeutic effects of activation of TNFR2 and inhibition of TNFR1 should be explored further to more fully understand the mechanisms of the NBM in mediating cognition. With a specific focus on targeting TNFRs in therapeutic strategies possibly provide the promising effects of current AD drugs.

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Chapter 6

Immunization with Small Amyloid β-derived Cyclopeptide Conjugates Diminishes Amyloid -β-induced Neurodegeneration in Mice

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ABSTRACT

Background: Soluble oligomeric (misfolded) species of amyloid-beta (Aβ) are the main mediators of toxicity in Alzheimer's disease (AD). These oligomers subsequently form aggregates of insoluble fibrils that precipitate as extracellular and perivascular plaques in the brain. Active immunization against Aβ is a promising disease modifying strategy. However, eliciting an immune response against Aβ in general may interfere with its biological function and was shown to cause unwanted side-effects. Therefore, we have developed a novel experimental vaccine based on conformational neo-epitopes that are exposed in the misfolded oligomeric Aβ, inducing a specific antibody response. **Objective:** Here we investigate the protective effects of the experimental vaccine against oligomeric Aβ(1-42)-induced neuronal fibre loss *in vivo*. **Methods:** C57BL/6 mice were immunized or mock-immunized. Antibody responses were measured by enzyme-linked immunosorbent assay. Next, mice received a stereotactic injection of oligomeric Aβ(1-42) into the nucleus basalis of Meynert (NBM) on one side of the brain (lesion side), and scrambled Aβ(1-42) peptide in the contralateral NBM (control side). The densities of choline acetyltransferase-stained cholinergic fibres origination from the NBM were measured in the parietal neocortex postmortem. The percentage of fibre loss in the lesion side was determined relative to the control side of the brain. **Results:** Immunized responders (79%) showed 23% less cholinergic fibre loss ($p = 0.01$) relative to mock-immunized mice. Moreover, fibre loss in immunized responders correlated negatively with the measured antibody responses ($R^2 = 0.29$, $p = 0.02$). **Conclusion:** These results may provide a lead towards a (prophylactic) vaccine to prevent or at least attenuate (early onset) AD symptoms.

6.1. INTRODUCTION

With worldwide progressing aging populations the burden and prevalence of Alzheimer's disease (AD) is rapidly increasing. Over the next years AD may constitute an epidemic with high levels of individual suffering and staggering health care costs (Brookmeyer et al., 2007). AD is characterized by its pathological features of extracellular amyloid β (Aβ) plaques and tau protein aggregates known as intracellular neurofibrillary tangles (NFTs). Although characteristic for AD, it is generally believed that both Aβ plaques and NFTs are relatively non-toxic. Indeed, Aβ plaque load does not directly correlate with clinical symptoms of patients (Nelson et al., 2012; Smith and Perry, 1997). Instead, soluble oligomeric forms of Aβ are believed to be the main mediators of toxicity in AD (Ashe and Aguzzi, 2013; Glabe, 2008; Haass and Selkoe, 2007; Klein et al., 2004; Wisniewski and Goñi, 2015). These oligomeric species subsequently form aggregates of insoluble fibrils that precipitate as extracellular and perivascular plaques in the brain. In addition, intracellular aggregates of phosphorylated tau protein are formed at a later stage of the disease (Choi et al., 2014; Oddo et al., 2006). Based on this sequence of events it seems consequent to consider an immunization strategy directed against epitopes specific for the misfolded oligomeric forms of Aβ. Since 1999 (Schenk et al., 1999), there has been considerable interest in passive or active immunization with Aβ or Aβ-derived constructs against Alzheimer's disease, although a breakthrough has not yet been reached (Agadjanyan et al., 2015; Delrieu et al., 2014; Fettelschoss et al., 2014; Giacobini and Gold, 2013; Lemere, 2013; Mandler et al., 2015; Panza et al., 2014).

In a previous study, we showed that tetanus toxoid (TTd) conjugates of small cyclic peptides derived from amyloid Aβ, in particular cyclo[Aβ(22-28)-YNGK']-TTd, induced antibodies in Balb/c mice that cross-react *in vitro* with oligomeric and fibrillar Aβ(1-42) and amyloid plaques in mouse and human brain tissue. The antibodies did not recognize the homologous unmodified linear peptide or the amyloid precursor protein, indicating a high specificity for misfolded Aβ (Hoogerhout et al., 2011).

Testing the neuroprotective effect of AD related vaccines or compounds in *in vivo* models is rather complicated and in most cases indirect. Mutant mice with phenotypes resembling AD pathology often do not show an overt neurotoxicity and the protective function of compounds or vaccines can only be evaluated by indirectly measuring the postmortem plaque load or by analyses in living mice such as electrophysiological changes and/or cognitive/behavioral changes. In the present study, we have investigated if immunization can induce protection against acute oligomeric Aβ(1-42)-induced neurodegeneration of the cholinergic nucleus basalis magnocellularis of Meynert (NBM) in C57BL/6 mice. This *in vivo* model combines several advantages for testing compounds in proof of concept studies and reflects some essential hallmarks of AD pathology. The NBM is one of the early regions to be affected during AD and the overall neuropathology in this model, including cholinergic denervation and microglial activation, features much of the pathology found in AD patients (Granic et al., 2010a). The NBM lesion model provides the means to test directly the neuroprotective effect of the active immunization using cyclo-

Aβ peptides. Cholinergic fibres emanating from the NBM innervate cortical and hippocampal brain areas. Loss of this innervation can be quantified by measuring fibre density and used to determine neuroprotective strategies in AD (Dolga et al., 2009; Granic et al., 2010a; Luiten et al., 1995). Injecting a validated amount of neurotoxic oligomeric Aβ(1-42) into the NBM creates an acute lesion with ~40% loss of cholinergic neurons (Granic et al., 2010a). Here we provide evidence that active immunization using a trivalent vaccine consisting of cyclo[Aβ(22-28)-YNGK'], cyclo[Aβ(23-29)-YNGK'], and cyclo[Aβ(22-29)-YNGK'] peptide TTd conjugates provides significant protection in the Aβ(1-42)-induced NBM lesion model.

6.2. MATERIALS AND METHODS

6.2.1. Materials

Lyophilized human Aβ(1-42) (DAEFRHDSGY^{10}EVHHQKLVFF^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40}IA) was purchased from AnaSpec (Freemont, CA, USA) or rPeptide (USA). Scrambled-sequence Aβ(1-42) was purchased from rPeptide (USA). Monophosphoryl lipid A (MPL) was purchased from Sigma-Aldrich (Lipid A, monophosphoryl from *Salmonella enterica* serotype minnesota Re 595, Re mutant). An aqueous suspension of aluminum hydroxide (Alhydrogel 2%, 10.3 mg Al^{3+}/ml) was purchased from Brenntag (Frederikssund, Denmark), and monoclonal antibody 6E10 against Aβ(1-17) from Covance Research Products, Dedham, MA, USA). Tetanus toxoid (TTd) was produced by the former Vaccine Division of the National Institute for Public Health and the Environment (Bilthoven, The Netherlands).

6.2.2. Animals and Housing

Experiments were performed using 33 male C57BL/6JRj mice (Janvier, Le Genest-St-Isle, France), 6-8 weeks old at arrival. All mice were housed individually in macrolon type II cages (length 35 cm, width 15 cm, height 13.5 cm, Bayer, Germany), with sawdust as bedding and shredded cardboard as nesting material. The mice were kept in a climate room with controlled temperature (22 ± 1°C) and humidity (55 ± 10%). A light/dark (LD) schedule (12h light - 12h dark; lights on at 07:00 h GMT + 1h; ± 50 lux) was maintained. Food (standard rodent chow: RMHB/ 2180, Arie Block BV, Woerden, NL) and normal tap water were available *ad libitum*. Cages were cleaned at least every two weeks. All mice were checked daily for food/water/health/activity/abnormal behavior. All procedures were in accordance with the regulation of the ethical committee for the use of experimental animals of the University of Groningen, The Netherlands (License number DEC 6579A).

6.2.3. Experimental Outline

Mice were habituated to the climate room and housing conditions for seven days before receiving the first round of immunization. The mice were immunized by subcutaneous injection in the groin with either our trivalent vaccine (referred to as 'immunized' mice, n = 24) or mock vaccine (referred to as 'mock-immunized' or 'mock' mice, n = 9). Three, four or five immunizations were performed with three-weekly intervals. An overview of the experimental procedures is provided in Figure 6.1. Twelve days after all mice had received the third immunization, blood samples were collected from the tail vein and the derived serum samples were analyzed the next day (day 55) by enzyme-linked immunosorbent assay (ELISA). Based on the anti-oligomeric Aβ(1-42) endpoint titers obtained (ELISA 1), 8 immunized and 3 mock-immunized mice were selected for stereotactic Aβ(1-42)-induced NBM lesion surgery which was performed the next day (group A, day 56, two weeks after the third immunization). Remaining mice received a fourth immunization (on day 63) and another tail puncture after 12 days (on day 75). Based on the titers obtained (ELISA 2), 8 immunized and 4 mock-immunized mice were selected for Aβ(1-42)-induced NBM lesion surgery (group B, day 77, two weeks after the fourth immunization). Remaining mice (group C: eight immunized, two mock-immunized mice) received a fifth immunization (on day 84) and received Aβ(1-42)-induced NBM lesion surgery two weeks later (day 98). Mice from each group were transcardially perfused 10 days after NBM lesion surgery. Final blood samples were collected from the heart just before perfusion and derived serum samples were analyzed later (ELISA 3). Brains were isolated for further immunohistochemical analysis.

6.2.4. Peptide Synthesis and Purification

The synthesis and purification of the cyclic peptides cyclo[Aβ(22-28)-YNGK'], cyclo[Aβ(23-29)-YNGK'], and cyclo[Aβ(22-29)-YNGK'], in which K' is N^{ε}-(*S*-acetylmercaptoacetyl)lysyl for conjugation purposes, was performed as described earlier (Brugghe et al., 1994; Hoogerhout et al., 2011).

6.2.5. Preparation of Conjugates and Vaccines

The peptides were coupled to either bromoacetylated TTd (to obtain vaccine components) or maleimidyl-modified bovine serum albumin (BSA) (to obtain ELISA coating antigens) D. Bromoacetylated TTd was also capped with 2-aminoethanethiol, without prior incubation with peptide, to obtain the protein for the mock vaccine (capped TTd).

6.2.6. Vaccine Preparation

Aliquots (300 or 120 µl) of a solution of MPL (1.0 mg/ml) in chloroform/methanol/water, 74/23/3, were evaporated to dryness in round bottom polypropylene tubes. MPL films obtained from 300 µg samples were reconstituted with 0.60 ml of each cyclopeptide-TTd conjugate (0.50 mg/ml in physiological salt), followed by 0.58 ml Alhydrogel. MPL films obtained from 120 µg samples were reconstituted with 0.72 ml of capped TTd (0.50 mg/ml in physiological salt), followed by 0.23 ml Alhydrogel. The tubes containing the

suspensions were left on a roller mixer for 1 h at room temperature and thereafter stored overnight at 4°C. Then, the concentrated vaccine was diluted with 6.62 ml of physiological salt and the concentrated mock vaccine with 2.65 ml physiological salt. One dose of 0.3 ml of vaccine contained 10 µg of each cyclopeptide-TTd conjugate, 10 µg MPL, and 0.2 mg Al^{3+}. One dose of 0.3 ml of mock vaccine contained 30 µg capped TTd, 10 µg MPL, and 0.2 mg Al^{3+}.

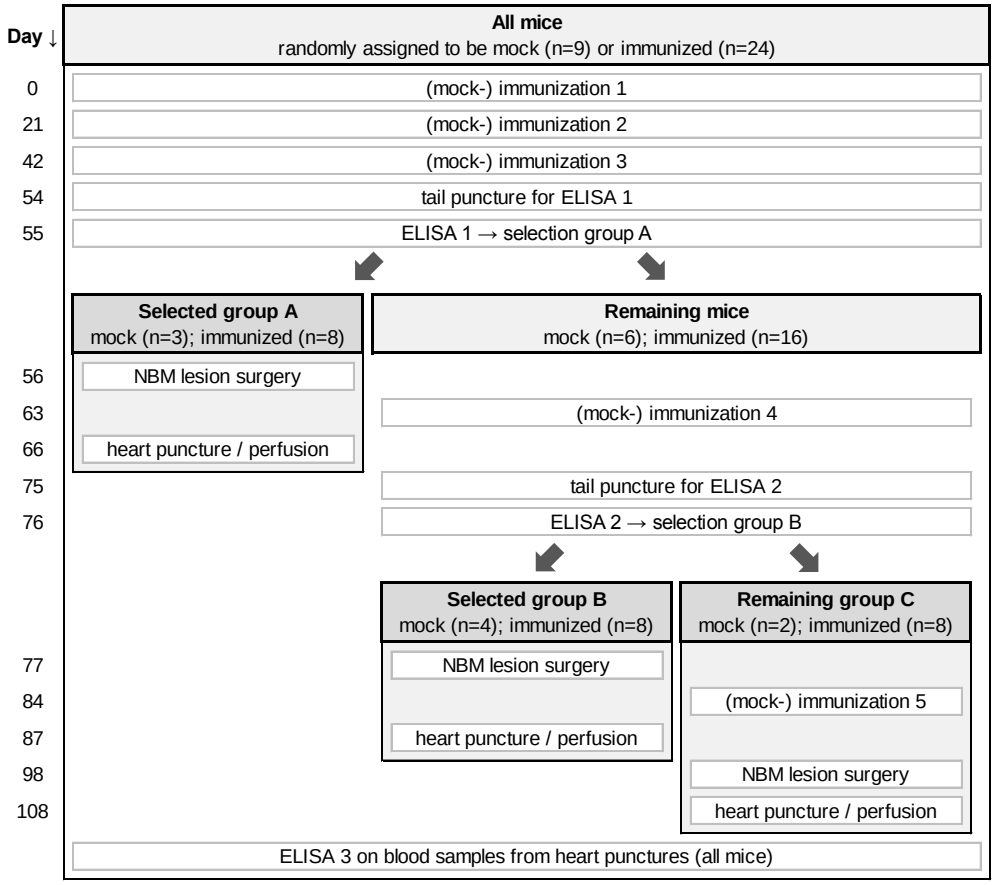

Figure 6.1. Experimental overview. Mice were randomly assigned to be mock-immunized (mock) or immunized. All mice received three rounds of subcutaneous injections in the groin with a 3-week interval. Mock mice received mock vaccine, immunized mice received our trivalent vaccine. Blood samples were collected from the tail vein and the serum was analyzed by ELISA. Based on the anti-oligomeric Aβ(1-42) endpoint titers obtained (ELISA 1), 8 well responding immunized mice and 3 mock mice were selected for oligomeric Aβ(1-42)-induced NBM lesion surgery (group A). Remaining mice received a fourth immunization and another tail puncture. Based on the titers obtained (ELISA 2), 8 immunized- and 4 mock mice were selected for NBM lesion surgery (group B). Remaining mice (group C: 8 immunized, 2 mock mice) received a fifth immunization before NBM lesion surgery. Mice

from each group were transcardially perfused 10 days after NBM lesion surgery. Final blood samples were collected from the heart just before perfusion and sera obtained were analyzed later (ELISA 3). Experimental days are indicated on the left.

6.2.7. ELISA

Wells of microtiter plates (Greiner 655092) were coated with oligomeric Aβ(1-42), peptide-BSA conjugates or TTd. Freshly prepared Aβ(1-42) oligomers were diluted to a final concentration of 2.5 μM (11.3 μg/ml) in 0.04 M sodium carbonate/bicarbonate buffer, pH 9.70. Peptide-BSA conjugates and TTd were diluted to a total concentration of 0.5 μg/ml in the same carbonate/bicarbonate buffer. Aliquots (100 μl) of these solutions were transferred into wells of the plates. The plates were incubated for 90 min at 37°C, emptied and washed twice with tap water containing 0.05% Tween 80. The plates were incubated with threefold serial dilutions of the serum samples in phosphate buffered saline containing 0.1% Tween 80. Monoclonal antibody 6E10 was used as positive control on plates coated with oligomeric Aβ(1-42). The starting dilution for determination of anti-oligomeric Aβ(1-42) titers and anti-linear[Aβ(1-15)-C] titers was 1/150. For determination of anti-cyclopeptide and anti-TTd titers, a starting dilution of 1/1500 was used. The plates were further processed as described earlier (Westdijk et al., 1997). Endpoint titers were defined as the dilution, which gave an optical density (OD) reading identical to the mean OD plus three times standard deviation of sera of mice immunized with mock vaccine (Frey et al., 1998).

6.2.8. Preparation of Oligomeric Aβ(1-42) for Stereotactic NBM Lesions

Oligomeric Aβ(1-42) was prepared as described before (Dahlgren et al., 2002; Granic et al., 2010b; Stine et al., 2003). In short, lyophilized Aβ(1-42) peptide (rPeptide, USA) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma-Aldrich, St. Louis, USA) at a concentration of 1 mM. The peptide solution was aliquoted and the HFIP removed by evaporation at room temperature in a flow cabinet for 3 h. The dry peptide films were stored at -20°C until further processing. Before use, Aβ(1-42) films were dissolved in anhydrous DMSO to 5 mM and subsequently diluted in neurobasal medium to a final concentration of 250 μM. The solution was incubated at 4°C for 24 h to enable Aβ(1-42) oligomerization. Scrambled Aβ(1-42)control peptide was processed using exactly the same procedure.

6.2.9. Stereotactic Aβ(1-42)-induced NBM Lesions

Mice were anaesthetized with an isoflurane/oxygen gas mixture and received 60 μl finadynes.c. (1 mg/ml, Schering-Plough NV/SA, Brussels, Belgium). Mice were then placed in a stereotact (Kopf instruments model 900, Tujunga, CA, USA) secured with cheek bars and a tooth-bar/nose clamp. The stereotact was equipped to support maintenance of the isoflurane/oxygen anesthesia. Eyes were protected against dehydration by applying Vita-Pos® salve (Ursapharm) and the shaved skin was disinfected with 70% ethanol. A small

medial incision was made from bregma to lambda. The periost was gently scraped away with a scalpel and the scalp was cleaned using cotton swaps. A hole was drilled in the skull and the dura was punctured with a needle. A 1-µl Hamilton syringe was slowly lowered into the brain (coordinates: 0.6 mm posterior to bregma; 2.1 mm lateral to the sagittal suture). 0.2 µl of 250 µM oligomeric Aβ(1-42) was slowly (0.1 µl/min) injected at 4.6 and 4.4 mm ventral to the dura (total unilateral injection amount: 0.4 µl, 100 pmol). The stereotactic coordinates had been confirmed by several pilot experiments in which trypan blue was injected into the NBM and traced (post-mortem) to the NBM. After injection, the syringe was kept in place for 2 min at each injection site to optimize diffusion into the brain and to limit spread of the solution during withdrawal of the needle. The same procedure was followed on the contralateral side of the brain, where scrambled Aβ(1-42) control peptide was injected. Left/right injections of the peptides were randomized between mice. After contralateral injection, mice were immediately removed from the stereotact. The head wound was sutured (Ethicon perma-hand N266 5-0), disinfected with Povidine-iodine (Betadine®) and mice received an i.p. injection of 0.5 ml warm (±25°C) saline/glucose (0.45% NaCl + 2.5% glucose). Mice were placed back in their homecage and kept under a IR heat lamp for 24 h.

6.2.10. Transcardial Perfusion

At postoperative day 10, under deep pentobarbital anaesthesia, mice were perfused transcardially for 1 min with 0.9% NaCl + 0.5% heparin (400 U) in H_2O (15 ml/min), followed by 150 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) for fixation. Brains were collected, post fixated for 24 h in 4% PFA in 0.1 M PB, rinsed for one day in 0.01 M phosphate buffered saline (PBS, pH = 7.40) and then kept overnight in 30% sucrose in PBS cryoprotectant at room temperature. Brains were frozen using liquid nitrogen and stored at -80^{o}C. The brains were cut in 20 µm coronal sections using a cryotome, collected in PBS (0.01 M, pH = 7.40) containing 0.1% sodium azide and kept at 4^{o}C until choline acetyltransferase (ChAT) immunostaining.

6.2.11. Immunohistochemical ChAT Staining

The free floating brain sections were rinsed three times (rinsing was always performed in 0.01 M PBS, pH = 7.40, for 5 min on a shaker) before being incubated for 30 min in 0.3% H_2O_2 in PBS. Next, the brain slices were rinsed five times before pre-incubation for 1 h at room temperature in PBS containing 5% normal rabbit serum (NRS, Zymed, San Francisco, CA, USA) and 0.4% Triton X-100. Subsequently, sections were incubated for 3 days at 4^{o}C in the primary antibody solution containing 1:333 diluted goat anti-ChATIgG (Millipore, Billerica, MA, USA), 1% NRS, 0.5% BSA and 0.4% Triton X-100 in PBS. Next, brain sections were rinsed five times and thereafter incubated at room temperature for 4h in the secondary antibody solution containing rabbit anti-goat IgG 1:500 (Sigma), 1% NRS, 0.2% Triton X-100 and 0.5% BSA in PBS. Afterwards, sections were rinsed five times before being incubated at RT for 2 h with Vectastain Elite ABC Kit (Vector Laboratories, CA, USA). Both the 'A' and 'B' solution were diluted to 1:500). The staining was completed with

nickel-enhanced (BDH Chemicals Ltd., UK) diaminobenzidine (DAB) reaction in the presence of H_2O_2. The next day, slices were mounted on glass from a 1% gelatin + 0.01% aluin solution, dried overnight and defatted/dehydrated through respectively 100% EtOH, 100% EtOH, 70% EtOH + 30% Xylol, 30% EtOH + 70% Xylol, 100% Xylol, 100% Xylol, 100% Xylol. Glass preparations were cover slipped using DPX mountant, dried for two days and then cleaned.

6.2.12. Quantitative Image Analysis

The parietal neocortex is topographically the target of the afferent cholinergic pathway from the NBM sites where the Aβ(1-42) oligomers were injected. The quantification procedure was established in our laboratory and described in detail in a series of previous publications (Dolga et al., 2009; Granic et al., 2010a; Harkany et al., 1998, 2001; Horvath et al., 2000). Briefly, exact measurement took place in the superficial sublayer of the layer V cortical area representing the densest zone of cortical cholinergic innervation (coordinates: 0.6 mm posterior to bregma; Paxinos and Franklin, 2001). After background subtraction and gray-scale threshold determination, the surface area of ChAT-positive fibres (percentage of the area covered by ChAT-positive cholinergic fibres relative to the total sampling area) was measured on both contralateral sides of 8 coronal sections per animal using a Quantimet 600 HR Image Analysis System (Leica, Rijswijk, the Netherlands). The percentage of fibre loss relative to the control side was calculated within each of the slices and these values were averaged as an indicator of cholinergic degeneration per animal. One immunized mouse showed over 40% fibre loss and was excluded as an outlier from all analyses.

6.2.13. Statistics

Statistical analyses were performed using GraphPad Prism 5.01 (GraphPad software, Inc.) and SigmaPlot 12.5 (Systat software, inc.). Fibre densities in control- and lesion sides of the brains of mock and immunized mice were analyzed using a 2-way repeated measures (RM) analysis of variance (ANOVA). Differences between groups were tested by two-tailed unpaired t-tests. Pre-post differences between groups were tested by two-tailed paired t-test. Correlations were performed using Pearson correlations. *$p < 0.05$ was considered significant.

6.3. RESULTS

6.3.1. ELISA: Results before Aβ(1-42)-induced Nucleus Basalis Lesions

Mice were immunized with either trivalent vaccine (immunized, n = 24) or mock vaccine (mock, n = 9). Three, four or five (mock-) immunizations were performed with three-

weekly intervals while intermediate blood samples were taken from the tail vain (Figure 6.1). The derived serum samples were analyzed by enzyme-linked immunosorbent assay (ELISA) using oligomeric Aβ(1-42) as coating antigen. Figure 6.2 shows the ^{10}log[Aβ(1-42) endpoint titers] obtained. Vertical axis categories are numbered between square brackets for easy reference.

After three immunizations (ELISA 1 in Figure 6.1), a significant difference was found between all mock and immunized mice (Figure 6.2 [1]: two-tailed unpaired t-test: $p < 0.001$).

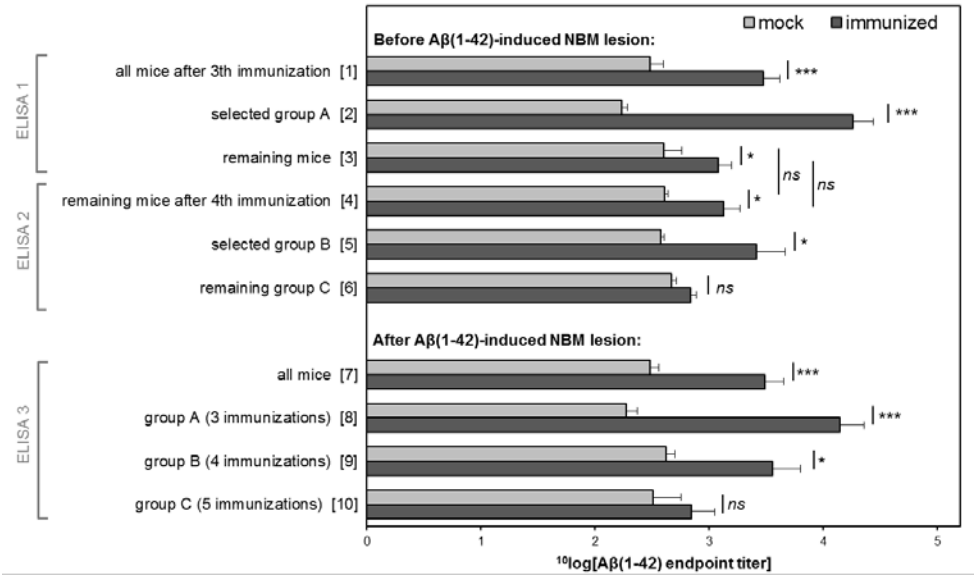

Figure 6.2. Average of the ^{10}log value of anti-oligomeric Aβ(1-42) ELISA endpoint titers of immunized and mock mice before- and after Aβ(1-42)-induced NBM lesions. After all mice had received three (mock-) immunizations, mice with high titers (ELISA 1) and randomly selected mock mice were selected as the first group to receive NBM lesion surgery (group A). Remaining mice received a fourth (mock-) immunization. Again, mice with high titers (ELISA 2) and randomly selected mock mice were selected as the second group to receive NBM lesion surgery (group B). Remaining mice (group C) received a fifth (mock-) immunization before lesion surgery. Measurements before NBM lesions (ELISA 1 and 2) were performed on sera from blood samples collected from the tail vein. Measurements after NBM lesions (ELISA 3) were performed on sera from blood samples taken from the heart, just before transcardial perfusion. The results show that three rounds of immunizations are highly effective, while a fourth and fifth round of immunizations have only minor added effect. Vertical axis categories are numbered between square brackets for easy reference. Error bars represent SEM. Statistical indicators: * $p < 0.05$, *** $p < 0.001$, *ns* indicates $p > 0.05$.

Mice (n = 8) with high titers were selected for the first round of Aβ(1-42)-induced NBM lesion surgery, forming 'group A' together with randomly selected mock animals (n = 3)

(Figure 6.2 [2]: two-tailed unpaired t-test mock vs. immunized mice: $p < 0.001$). Remaining animals still showed a significant difference between mock and immunized mice (Figure 6.2 [3]: two-tailed unpaired t-test: $p < 0.05$), but received a fourth (mock-) immunization followed by a second tail vein puncture. Aβ titers (ELISA 2) showed that the difference between mock and immunized mice was maintained (Figure 6.2 [4]: two-tailed unpaired t-test: $p < 0.05$), but that the fourth immunization did not significantly increase Aβ titers (Figure 6.2 [4] vs. [3]: two-tailed paired t-test before vs. after fourth immunization: mock: $p = 0.98$, immunized: $p = 0.81$). Again, immunized mice with high titers ($n = 8$) and randomly selected mock mice ($n = 4$) were selected for the second round of Aβ(1-42)-induced NBM lesion surgery, forming group B (Figure 6.2 [5]: two-tailed unpaired t-test mock vs. immunized mice: $p < 0.05$). The remaining mock ($n = 2$) and immunized ($n = 8$) mice formed group C. As measured from the serum samples after the fourth immunization (ELISA 2), no significant difference between mock and immunized animals was present in group C mice (Figure 6.2 [6]: two-tailed unpaired t-test: $p = 0.46$). These mice received a fifth round of (mock-) immunization before Aβ(1-42)-induced NBM lesion surgery.

6.3.2. ELISA: Results after Aβ(1-42)-induced Nucleus Basalis Lesions

After the Aβ(1-42)-induced NBM lesions, blood samples from all mice were obtained by a heart puncture just after the mice were sacrificed, before transcardial perfusion. ELISA analysis (ELISA 3) showed that considering all animals, a significant difference was again found between mock and immunized mice (Figure 2 [7]: two-tailed unpaired t-test: $p < 0.001$). Similarly, a significant difference was again found between mock and immunized mice of group A and B, but not of group C (Figure 6.2 [8], [9], [10]: two-tailed unpaired t-test: group A: $p < 0.001$; group B: $p < 0.05$; group C: $p = 0.46$). These results confirm the earlier intermediate titers before the Aβ(1-42)-induced NBM lesions (ELISA 1 and 2).

No significant differences were found between the titers before vs. after the Aβ(1-42)-induced NBM lesions of mock and immunized mice (statistical comparisons not indicated in Figure 6.2: two-tailed paired t-test, before vs. after NBM lesion: group A mock mice: $p = 0.42$; group A immunized mice: $p = 0.33$; group B mock mice: $p = 0.94$; group B immunized mice: $p = 0.30$; group C mock mice: $p = 0.67$; group C immunized mice: $p = 0.96$). These results indicate that injecting oligomeric Aβ(1-42) in the NBM, did not significantly affect antibody responses. Moreover, these results indicate that the fifth round of (mock-) immunizations, as performed on the group C animals, did not significantly increase their average antibody response. Together, the results show that three rounds of immunizations are very much effective, while a fourth and fifth round of immunizations have only minor added effect.

6.3.3. ELISA: Responders and Non-responders

The ELISA results revealed a degree of variation in the measured antibody responses. Although most mice responded well to the immunizations, others showed a lesser response. Supplementary Table I shows the ^{10}log[Aβ(1-42) endpoint titers] obtained for

individual mice. We used a rigorous criterion to identify responders and non-responders. 'Responders' were defined as mice which showed higher titers than the average of the mock animals plus three standard deviations. After three immunizations, 14 out of 24 mice (56%) were identified as responders (ELISA 1). Measurements on serum samples after the fourth immunization (ELISA 2) revealed both increases- and decreases in titers. Measurements from serum samples obtained after the heart puncture before transcardial perfusion (ELISA 3) showed 16 out of 24 responders (67%). Overall, 79% of the mice were identified as a responder in at least one measurement, leaving five mice (21%) identified as consistent non-responders.

6.3.4. ELISA: Investigating Vaccine Specificity

The sera obtained by heart puncture were analyzed more extensively (ELISA 3). In addition to oligomericAβ(1-42), BSA conjugates of cyclo[Aβ(22-28)-YNGK'] and its 23-29 and 22-29 analogues and linear [Aβ(1-15)-C], as well as plain TTd, were used as coating antigens. As expected, all serum samples obtained after the heart puncture had high anti-TTd antibody titers (all mice showed a ^{10}log[endpoint titer] > 5.52 on a TTd ELISA coating, data not shown). The serum samples of immunized mice showed high antibody titers against each of the three amyloid cyclopeptides, but not to linear Aβ(1-15) which contains the immunodominant epitope of Aβ(1-42) (data not shown).

6.3.5. Immunohistochemistry: Aβ(1-42)-induced Nucleus Basalis Lesions

An excitotoxic damage model was used to assess whether immunized mice were more protected against oligomeric Aβ(1-42) insult to the brain compared to mock mice. The nucleus basalis magnocellularis (NBM) is the origin of many cholinergic projections to the cortex. Oligomeric Aβ(1-42) was unilaterally injected in the NBM and the neurotoxic impact assessed by determining cholinergic fibre loss in the parietal neocortex relatively to the control hemisphere which was injected with scrambled Aβ(1-42) peptide (Aβ-scr) (Figure 6.3).

In the mock mice, average fibre density on the control- and lesion side was 10.30 ± 0.36 SEM and 7.95 ± 0.28 SEM respectively. In the immunized mice, average fibre density on the control- and lesion side was 9.91 ± 0.35 SEM and 8.17 ± 0.33 SEM respectively. A 2-way RM ANOVA with independent variables 'treatment' (mock-immunized or immunized) and 'brain side' (oligomeric Aβ(1-42)-injected or scrambled Aβ(1-42)-injected), revealed a significant difference between brain sides. The side of the brain injected with oligomeric Aβ(1-42) consistently showed less fibre density than the scrambled-Aβ injected sides of the brains ($F(1,30) = 398.83$, $p < 0.001$) (Figure 6.4A). An 'All Pairwise Multiple Comparison' posthoc test (Holm-Sidak method) revealed that this difference was present within both the mock- ($t = 13.54$, $p < 0.001$) and immunized mice ($t = 16.02$, $p < 0.001$).These results indicate that the oligomeric Aβ(1-42)-induced NBM lesion model was highly effective. A significant interaction effect was found between treatment and brain side ($F(1,30) = 8.88$, $p = 0.006$), but no significant effect of treatment was detected ($F(1,30) = 0.02$, $p = 0.88$)

within either the oligomeric Aβ(1-42)-injected- (t = 0.38, p = 0.71), or scrambled Aβ(1-42)-injected (t = 0.67, p = 0.51) brain sides.

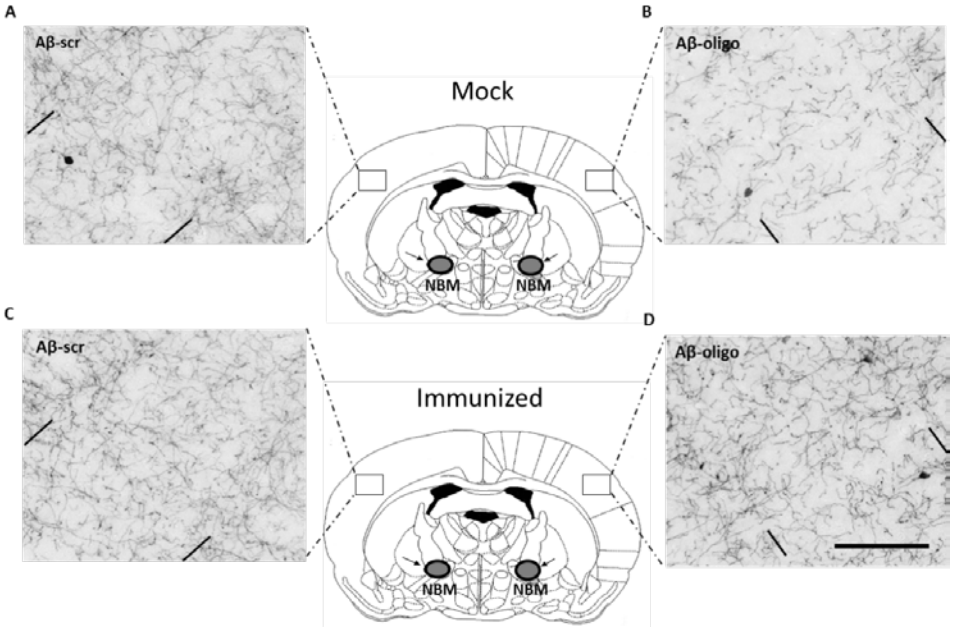

Figure 6.3. Active immunization prevents the loss of cholinergic innervations induced by oligomeric Aβ(1-42) injected into the NBM. Shown are representative images of ChAT-positive fibres in the parietal neocortex of mock (**A** and **B**) and immunized mice (**C** and **D**). Images on the left (A and C) show the side of the brain injected with scrambled Aβ(1-42) control peptide (Aβ-scr). Images on the right (B and D) show the contralateral lesion-sides of the brain injected with oligomeric Aβ(1-42) (Aβ-oligo). Note that in practice the compounds were randomly injected in the left- or right side of the brain. Comparing control- and lesion sides of the brain, immunized mice show reduced fibre loss compared to mock mice. In each image the area between the parallel bars indicates the quantified area (layer V of the somatosensory cortex). The horizontal scale bar shown in (D) applies to all images and represents 100 µm.

However, differences between mock and immunized mice became apparent when comparing lesion sides with control sides within each of the brain slices and calculating the average percentage of fibre loss (Figure 6.4B). On average, immunized mice showed significantly less loss of cholinergic fibres in the parietal neocortex than mock mice (immunized: 16.77 % ± 0.99 SEM vs. mock: 21.05 % ± 1.43 SEM, two-tailed unpaired t-test: p = 0.02, effect size: Cohen's d = 0.95). As expected, these statistics slightly improve when excluding the five identified non-responders from the immunized group (immunized responders: 16.22 % ± 1.09 SEM vs. mock: 21.05 % ± 1.43 SEM, two-tailed unpaired t-test: p = 0.01, effect size: Cohen's d = 1.08). Immunized mice showed 20.3% reduced fibre loss

relative to the mock mice, or 22.9% when excluding the five non-responders from the immunized mice.

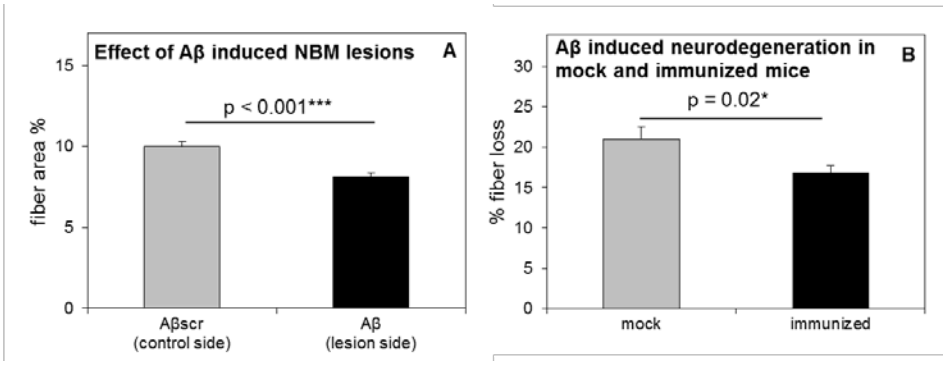

Figure 6.4. Effectiveness of the oligomeric Aβ(1-42)-induced NBM lesion model and effects in mock and immunized mice. All mice consistently showed less cortical cholinergic fibre density on the Aβ injected side of the brain, as compared to the scrambled-Aβ (Aβscr) injected control side of the brain (**A**). Compared to mock mice, immunized mice showed significantly reduced cholinergic fibre loss in the cortex, as measured by optical fibre density after ChAT immunostaining (**B**). In both panels the error bars represent SEM.

6.3.6. Correlation between Cholinergic Fibre Loss and Antibody Response

We investigated whether the loss of cholinergic fibre density in the cortex correlated negatively with the measured antibody responses. When using the Aβ(1-42) endpoint titers based on the serum samples obtained after the heart puncture (ELISA 3), a significant negative correlation was found between the titers and the percentage of fibre loss when considering all mice (Pearson r = -0.38, R^2 = 0.15, p = 0.03). However, this correlation was not significant when considering only the immunized mice, with or without the five non-responders (p = 0.49 and p = 0.91 respectively). The titers obtained from the tail vain puncture before Aβ(1-42)-induced NBM lesions, after all mice had equally received three immunizations (ELISA 1), showed similar results with a better correlation. Using these titers a significant negative correlation was found between fibre loss and antibody response (considering all mice: Pearson r = -0.49, R^2 = 0.24, p = 0.004; considering only the immunized mice: Pearson r = -0.44, R^2 = 0.20, p = 0.03; considering immunized mice without the non-responders: Pearson r = -0.54, R^2 = 0.29, p = 0.02). Results are shown in Figure 6.5.

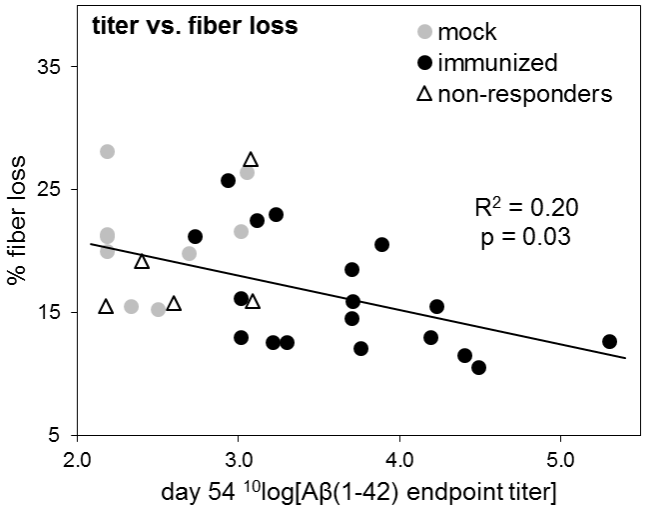

Figure 6.5. Correlation of the ^{10}log value of anti-oligomeric Aβ(1-42) endpoint titers (x-axis) vs. cholinergic fibre loss (y-axis). Titers were obtained by ELISA of sera obtained from blood samples taken from the tail vein on day 54 (ELISA 1), after which all mice had received three immunization injections. Percentage of fibre loss was calculated for each animal based on ChAT-stained cholinergic fibre density in the bilateral parietal neocortex, which is the target of afferent cholinergic pathways from the bilateral NBM sites. The Aβ(1-42) injected lesion side of the brain was compared to the scrambled-Aβ(1-42) injected control side of the brain to determine the percentage of cholinergic fibre loss. The linear regression line and Pearson correlation is based on the immunized mice (indicated by black circles) including the non-responders (separately indicated by open triangles), but not including the mock mice (indicated by grey circles). The R-squared- and two-tailed p-value is indicated in the figure.

6.4. DISCUSSION

In recent years it became more and more apparent that amyloid plaques do not primarily correlate with AD pathology. This seems rather be true for the amount of tangles consisting of hyperphosphorylated tau proteins (Alafuzoff et al., 1987; Arriagada et al., 1992; Gómez-Isla et al., 1997; Herrup, 2015; Musiek and Holtzman, 2015; Wilcock and Esiri, 1982). A possible explanation for that may lie in the fact that Aβ plaques *per se* are not neurotoxic and that tangles in contrast represent dead neuronal cell bodies and therefore neuronal cell loss. In fact the most neurotoxic molecules in AD pathology turned out to be soluble oligomeric forms of misfolded Aβ (Haass and Selkoe, 2007; Lue et al., 1999; McLean et al., 1999; Näslund et al., 2000; Wang et al., 1999). As even under normal physiological conditions Aβ is produced (Moghekar et al., 2011), a vaccination against non-

oligomeric or non-fibrillary Aβ might result in unwanted side effects (Tabira, 2010). Indeed, non-aggregated Aβ peptide at low doses have been found to exhibit neurotrophic effects including enhancement of neuronal survival, neurite-promoting and neural stem cell differentiation effects (Heo et al., 2007; Pike et al., 1993; Whitson et al., 1989, 1990; Yankner et al., 1990). Using cyclic peptides mimicking conformational neo-epitopes of oligomeric Aβ forms could circumvent the problems that would arise if peptides would be used which represent epitopes related to Aβ in general.

In a previous study, we have shown that tetanus toxoid (TTd) conjugates of small cyclic peptides derived from Aβ, in particular cyclo[Aβ(22-28)-YNGK']-TTd can be used for active immunization in mice. Two immunizations effectively produced antibodies in Balb/c mice that cross-react *in vitro* with oligomeric and fibrillar Aβ(1-42) and amyloid plaques in mouse and human brain tissue. Importantly, the antibodies did not recognize the homologous linear peptide or the amyloid precursor protein, indicating a high specificity for misfolded Aβ (Hoogerhout et al., 2011). In preliminary experiments it was found that C57BL/6 mice responded rather poorly to the original vaccine formulation, that is, cyclo[Aβ(22-28)-YNGK']-TTd conjugate with aluminum phosphate as adjuvant. The response in C57BL/6 was improved by immunization with the trivalent vaccine as used in the current study (a mixture of TTd conjugates of three cyclopeptides, cyclo[Aβ(22-28)-YNGK'] and its 23-29 and 22-29 analogues, with aluminum hydroxide gel and monophosphoryl lipid A (MPL) as adjuvant). In addition, a third immunization significantly improved the antibody response (data not shown). Our current data confirm these earlier results and show that subsequent fourth and fifth round of immunizations have little added effect on the antibody response. It is noteworthy that in the course of our investigation it was reported that the MPL adjuvant by itself may have beneficial effects on AD pathology, as measured in APPswe/PS1 transgenic mice (Michaud et al., 2013; Wang, 2013). In the current study both immunized and mock-immunized mice received the same amounts and dosages of MPL adjuvant. Therefore, the mock mice might have had some level of protection, induced by the MPL they received, indicating that the effect of immunization might be underestimated in the current study. Future studies may lead to further optimization of the vaccine formula, dosage, and administration scheme to enhance its efficacy.

We used the established NBM lesion model in C57Bl/6J mice (Dolga et al., 2009; Granic et al., 2010a), to test directly whether the specific antibodies produced by the active immunization have a protective potential against the cytotoxic effects of oligomeric Aβ(1-42). We found that immunized mice showed 20% less cholinergic fibre loss in the parietal neocortex relative to mock-immunized mice (or 23% when excluding five non-responders).

These results are very promising. Injecting substantial amounts of neurotoxic oligomeric Aβ(1-42) into the NBM creates an acute lesion. It was not certain whether the used Aβ concentration and incubation time (10 days) were optimal to show an effect at all. Similarly, it was uncertain if there would be enough neutralizing antibodies produced by the immune system and if these antibodies would reach the lesion site effectively enough.

Although we did not attempt to detect (in brain slices) the specific anti-Aβ antibodies produced by the immune system, it has been shown that peripheral antibodies are able to cross the blood brain barrier (Sas et al., 2008). Additionally, due to the administration of Aβ(1-42) via stereotactic injection, a local microglial response may have led to a local leakage of the blood brain barrier, allowing anti-Aβ antibodies to enter the brain parenchyma from blood vessels.

A main source of variation was the observed difference in antibody response of the mice. This variation allowed us to correlate the antibody responses of individual mice to the measured fibre loss. As expected, fibre loss significantly correlated negatively with the antibody titers induced by immunization. When considering only the immunized mice, this was however only true when using the titers obtained after all mice had received three rounds of immunizations (ELISA 1, before Aβ(1-42)-induced NBM lesions), but not when using the titers obtained from the serum samples after the NBM lesions (ELISA 3). Note that the blood samples taken after three rounds of immunizations (ELISA 1) were collected from all mice at the same time, while the heart punctures (ELISA 3) were acquired and processed at different days for the three groups (see supplemental table I). The serum samples obtained after three rounds of immunizations (ELISA 1) are therefore comparable measures and this initial assessment of the antibody response showed to correlate significantly with the measured fibre loss, even within the immunized group only and excluding consistent non-responders. These results are promising given that titers are not an accurate measure for effective antibody passage over the blood brain barrier and for the effectiveness of the immune system to neutralize Aβ.

Taken together, the tested trivalent vaccine has now been demonstrated to effectively induce a specific antibody response against misfolded Aβ in Balb/c as well as C57BL/6 mice without noticeable side effects. Furthermore, in the present study we show that this active immunization protects against acute oligomeric Aβ(1-42) insult to the brain *in vivo*. A clear next step is to immunize transgenic Alzheimer disease model mice and investigate the effect of immunization on cognition and amyloid plaque load with aging. Together, these results may open up the way to vaccination at an early stage of AD symptoms or before.

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Chapter 7

Summary and General Discussion

7.1 TNF-ALPHA SIGNALLING IN NEURODEGENERATION

Neurodegeneration is a detrimental process associated with neuronal damage and ultimately neuronal death in the central nervous system (CNS) involved in many diseases such as Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD), stroke, as well as trauma and brain ischemia. In particular, AD is the major common chronic disease with progressive neuronal death. The main pathological findings in AD include molecular changes, such as amyloid-β peptide plaque generation and tau protein hyperphosphorylation forming neurofibrillary tangles. Besides those changes, neuroinflammation is another major pathological feature of AD and notably characterized by activated microglia. Activated microglia are generally considered as immune cells in the brain, which can secrete a variety of cytokines that can be either trophic or toxic for neurons, dependent on the environmental conditions. One of the most important cytokines secreted by microglia is the pro-inflammatory cytokine TNF-α, which has been implicated to be involved in huge numbers of diseases, such as AD, excitotoxic injury, and MS (Gregory et al., 2012; Zheng et al., 2016).

TNF-α is synthesized as a transmembrane protein (mTNF-α) that can be processed by proteolytic cleavage via TNF alpha converting enzyme (TACE/ ADAM17) to a soluble homo-trimeric molecule (sTNF-α). TNF-α induces various cellular responses through its interaction with two distinct transmembrane receptors, TNFR1 and TNFR2. Under normal physiological conditions, TNFR1 is ubiquitously expressed in various cell types and tissues, whereas TNFR2 is predominantly expressed at low levels in immune cells and endothelial cells (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990; Cabal-Hierro and Lazo, 2012). TNFR1 activation can be induced by either sTNF-α or mTNF-α, and TNFR2 activation is predominantly initiated by mTNF-α (Grell et al., 1995). TNFR1 activation can trigger fibroblast growth, endothelial cell adhesion, neuroinflammation, and cell death while TNFR2 promotes proliferation of thymocytes and peripheral T cells and inhibition of early hematopoiesis (MacEwan, 2002).

Expression of TNF-α and TNFR1 is significantly up-regulated in the affected brain regions of AD patients and related with the pathopysiology of AD (Yamamoto et al., 2007; Zhao et al., 2003; He et al., 2007; Scalzo et al., 2009). Fiore et al. (2000) also showed that TNF-α over-expression in mice induced detrimental effects for synaptic plasticity led to memory and learning damages. Recently, TNF-α signalling pathways has been reported to associate with APP processing and Aβ production *in vitro* (Yamamoto et al., 2007). These studies indicate that anti-TNF-α reagents seem to be able to prevent or treat neurodegenerative disorders. In preliminary trials in rheumatoid patients anti-TNF appears to have an impressive effect on indices of disease activity including serum amyloid A production (Maini et al., 1993). Some anti-TNF-α agents, such as Lenercept, were applied to treat patients with rheumatoid arthritis and indeed effectively attenuates this inflammatory disease (Meyer, 2000). Anti-TNF-α therapeutics, however, failed in the CNS diseases (Arnason, 2011). This failure could be caused by TNF-α pleiotropic functions through its interaction with two distinct receptors: TNF-α binding to TNFR2 mediates neuroprotection in neurodegenerative diseases (Masuch et al., 2016; Sullivan et al., 1999;Barger et al.,

1995; Cheng et al., 1994; Fontaine etal. 2002; Marchetti et al., 2004; Dolga et al., 2008; Dong et al., 2015), whereas its interaction with TNFR1 triggers severe inflammation and thereby leads to cell death (Kaczmarek et al., 2013). Complete blockade of TNF-α therefore prevents not only TNFR1 mediated neuroinflammatory and degenerative but also TNF-α/TNFR2-mediated neuroprotective effects.

Expression of soluble TNFR levels (sTNFR1 and sTNFR2) are significantly elevated in cerebrospinal fluid and serum during preclinical stages of AD (Jiang et al., 2011). Production of sTNFRs correlate positive with TACE1 activity and Aβ40 levels and expression of tau protein (Buchhave et al., 2010). This seems that sTNFRs could be one of hallmarks of AD. However, sTNFRs circulate during inflammation and can neutralize TNF-α-induced cytotoxicity and immunoreactivity *in vivo* and *in vitro* (Van Zee et al., 1992). The role of sTNFRs has been detailed in Chapter 2 of this thesis. Whether sTNFRs could be applied to modulate inflammation-induced exacerbation in neurodegenerative disorders could be subject of further investigation. Nevertheless, sTNFRs could be considered as independent inflammatory markers, since the expression of TNFRs and their conversion into soluble forms is a complex process that not solely depends on TNF-α activity but also on other inflammatory effectors such as interferon-γ (Diez-Ruiz et al., 1995).

In Chapter 2, we mainly reviewed the complex TNF-α signalling pathways and their involvement in neurodegenerative disorders as well as the potential therapeutic properties resulting from the antithetic effects of TNFR1 and TNFR2 signalling. We discussed that selective targeting TNFRs could be the basis of a novel and effective therapeutic strategy against neurodegenerative disorders. This addressed our next investigations concerning on the therapeutic functions of targeting TNFRs reagents in neurodegenerative mouse models. Due to TNFR1-induced neurodegeneration and TNFR2-mediated neuroprotection, one specific manipulation of TNF-α downstream pathways is to use pharmacological agents that only block TNFR1 signalling without altering the activation of TNFR2 signalling pathway or that activate TNFR2 signalling, for instance, a TNFR1 antagonist and a TNFR2 specific agonist.

7.2 TARGETING TNFRS AS A THERAPEUTIC STRATEGY AGAINST ALZHEIMER'S DISEASE

Up-regulated TNFR1 increases aggregation of Aβ peptides and Aβ formation in AD mouse models (He et al., 2007; Scalzo et al., 2009), and its activation triggers severe inflammation and thereby leads to neuronal cell death (Kaczmarek et al., 2013). Additionally, it has been suggested that TNF-α mediates Aβ oligomer-induced memory impairments by interaction with its receptor one (Lourenco et al., 2013). He et al. (2007) found that deletion of TNFR1 diminishes Aβ plaque formation, inhibits Aβ generation and prevents learning and memory deficits in APP23 transgenic mice, indicating that inhibiting TNFR1 pathway could be a possible therapeutic approach in AD. Selective inhibitors of soluble TNF-α have been

demonstrated to efficiently prevent acceleration of amyloid plaques in transgenic mice by inactivation of TNF-α/TNFR1 signalling pathway (McAlpine et al., 2009). In contrast, TNFR2 activation has been implicated to mediate neuroprotection through PKB/Akt-mediated NF-κB pathway (Marchetti et al., 2004). TNFR2 null mice show sensitized response to cholesterol-bearing pullulan nanogel-induced resorption compared to wild type mice, suggesting also a protective role of TNFR2 (Nagano et al., 2011). Furthermore, Fontaine et al. (2002) reported that TNFR2 activation can protect against ischemic reperfusion-induced retinal neuronal death, which is in contrast with the role of TNFR1 signalling in this injury model. Recently, more evidence came up for the potentially protective effects of TNFR2 pathways. For instance, Fischer et al. (2011) demonstrated that agonistic TNFR2 activation significantly prevents neuronal cell death induced by oxidative stress; Aoki's group (2007) designed a loop peptide mimetic of TNFR1 that can inactivate TNFR1 signalling pathway and found that administration of this molecule strongly ameliorates collagen-induced arthritis. Williams and colleagues (Williams et al., 2014) have demonstrated that a TNFR1 antagonist attenuates MS-related pathological features in EAE mouse model; Furthermore, a novel TNFR1 antagonist effectively suppressed arterial inflammation and intimal hyperplasia in mice (Kitagaki et al., 2012). Therefore, these studies suggest that targeting TNFRs by activation of TNFR2 and/or inhibition of TNFR1 could become a therapeutic strategy against neurodegeneration, such as AD.

To investigate the therapeutic potential of humanized TNFR1 selective antagonists or humanized TNFR2 specific agonists *in vivo*, we generated knock-in mice that carry the human extracellular domains of TNFR1 and TNFR2. In Chapter 3, we demonstrated the generation and identification and characterization of knock-in mice with chimeric humanized TNFRs.

In Chapter 4, the therapeutic potential of both the human TNFR2 selective agonists, EHD2-scTNF$_{R2}$ and TNC-scTNF$_{R2}$, as well as the human specific TNFR1 antagonist ATROSAB were analyzed in the nucleus basalis lesion model. In this lesion model, NMDA was injected into the nucleus basalis magnocellularis (NBM) and thereby led to excitotoxic cell death of cholinergic neurons. This leads not only to a strong lesion in the NBM but also to a decline in cholinergic innervations in the parietal cortex of the brain. We found that EHD2-scTNF$_{R2}$ and TNC-scTNF$_{R2}$ as well as ATROSAB significantly reduced microglial activation at the site of the lesion and protected cholinergic neurons and the neocortical innervations against NMDA induced excitotoxic damage. Because the NBM lesion-mediated neocortical cholinergic denervation results in memory dysfunctions such as learning and memory deficits and retention dysfunctions (Coyle et al., 1983), we here evaluated the behavioral functions, associated with hippocampus, amygdala and cerebral cortex, affected by NBM-induced cholinergic denervation. The functional outcome after NMDA induced neurodegeneration as shown in Chapter 5 is in the line with tissue damage: impairment to the NBM selectively affected neocortical cholinergic innervation and its related retention memory deficits, while leaving in particular hippocampal and amygdala innervation and functions unaffected. These results confirmed that selective activation of TNFR2 and inhibition of TNFR1 as a therapeutic target could be highly useful to treat neurodegenerative conditions and diseases.

Importantly, we found that ATROSAB neuroprotection against NMDA-mediated excitotoxicity was dependent on a concurrent activation of TNFR2 signalling. This finding supports previous studies (Dolga et al., 2008a; Dolga et al., 2008b; Fontaine et al., 2002; Marchetti et al., 2004), which indicated the important protective signalling pathway of PI3 Kinase induced PKB/Akt phosphorylation and subsequent NF-κB activation through TNFR2 both *in vitro* and *in vivo*.

So far, our investigation thoroughly explained TNF-α duality on neuroprotection and aggravation in the CNS diseases, mainly due to its two distinct receptors. With TNFR2 being neuroprotective, whereas TNFR1 promotes neuronal degeneration and is involved in the pathology of AD by promoting Aβ generation. Furthermore, our results also provide the rational for the previous failure of clinical studies using anti-TNF-α drugs in neurodegenerative diseases and highlight the essential protective role of TNFR2. Our studies were also supported by recent studies using the biological compound XPro-1595 as a selective blocker of soluble TNF-α (Brambilla et al., 2011; Taoufik et al., 2011).

7.3 MODULATION OF AMYLOID-BETA

According to 'amyloid hypothesis', Aβ peptide accumulation is a pivotal pathological characteristic in the brain of AD patients. Various strategies therefore have been developed to minimize or reverse the negative effects of Aβ, for instance reducing Aβ load, inhibiting Aβ misfolding and aggregation, enhancing Aβ clearance (Scalzo et al., 2009; Schenk et al., 1999; Sigurdsson et al., 2000). Approaches diminishing Aβ level by inhibition of Aβ generation using β- and γ-secretase inhibitors, have experienced a drawback since these proteases are not only involved in the generation of Aβ but are as well involved in important cellular processes like axonal myelination, synapse maintenance and Notch signalling (Bittner et al., 2009; Lleó et al., 2003; Willem et al., 2006). Also, β-secretase inhibitors just reduce Aβ levels in the periphery but not in the brain (Georgievska et al., 2015), indicating that this strategy using β- or γ-secretase inhibitor is not sufficient. Thus, the challenge is to develop selective inhibitors that effectively target only the amyloidogenic cascade without affecting other cell processes.

Another approach for targeting Aβ-peptides is raising neutralizing antibodies by either passive or active immunization. Aβ vaccination has been shown already some time ago to lead to amyloid plaque clearance in a mouse model of AD (Schenk et al., 1999). Furthermore, immunization has also been demonstrated to reverse memory deficits in APP transgenic mice (Dodart et al., 2002). Vaccination against Aβ was considered to be the most promising therapeutic strategy until immunized patients showed a strong brain inflammatory response and a few eventually died (Imbimbo, 2002; Münch and Robinson, 2002). Potential safety concerns of anti-Aβ vaccines, lead to the development of alternative strategies to reduce Aβ burden in AD. Soluble oligomeric forms of Aβ are thought to be the chief mediator of cytotoxicity in AD (Wisniewski and Goñi, 2015).

Naturally secreted oligomeric assembly of Aβ potently alters hippocampal synaptic plasticity by inhibiting long-term potentiation which is associated with memory impairments (Walsh et al., 2002). Also, the number of dendritic spines was dramatically decreased when neurons were incubated with Aβ oligomers, but not with monomers (Walsh et al., 2002).

Therefore, immunotherapy could be achieved by the inhibition of Aβ aggregation using small peptides, which specifically recognize and interact with Aβ pathological forms to reduce its aggregation and toxicity. Vaccination by anti-oligomeric monoclonal antibodies shows improvements of cognitive functions by reducing Aβ deposition and tau pathology in 3xTg-AD mice (Rasool et al., 2013). A recent study showed that the Aβ-derived peptide prevents Aβ oligomers and protects against Aβ toxicity in transgenic C. elegans (Diomede et al., 2016). Of note, immunization with recombinant 6Aβ15-T antigen robust anti-Aβ serum, strongly recognize Aβ1-42 markedly reduced Aβ burden, and improved behavioral performance in different mice (Wang et al., 2016).

A previous study of Hoogerhout and colleagues showed that tetanus toxoid (TTd) conjugates of small cyclic peptides derived from Aβ, specifically cyclo[Aβ(22-28)-YNGK']-TTd, induced antibodies in Balb/c mice that cross-react *in vitro* with oligomeric and fibrillar Aβ1-42 and amyloid plaques in mouse and human brain tissue. The antibodies did not recognize the homologous unmodified linear peptide or the amyloid precursor protein, indicating a high specificity for misfolded Aβ (Hoogerhout et al., 2011). However, a poor response with this vaccine formulation was shown in C57BL/6 mice in preliminary experiments. They next developed a new vaccine that consisted of cyclo[Aβ(22-28)-YNGK'], cyclo[Aβ(23-29)-YNGK'], and cyclo[Aβ(22-29)-YNGK'] peptide with aluminum hydroxide gel and monophosphoryl lipid A (MPL) as adjuvant, assessed its potential immunization. Our research group tested the immunotherapeutic properties of this vaccine in C57BL/6 mice shown in Chapter 6 of the thesis. In the study, we established NBM lesion with oligomeric Aβ1-42 or scrambled Aβ1-42 in immunized and mock-immunized mice that both received the same amount of and dosages of MPL adjuvant. We found that C57BL/6 mice responded to this trivalent vaccine and there was a negative correlation between the antibody response and the cortical innervation loss. The MPL adjuvant by itself, therefore, may have beneficial effects on AD pathology, as measured in APP(swe)/PS1 transgenic mice (Michaud et al., 2013). In fact we could give supporting evidence for the protective function of an active vaccination against epitopes of oligomeric Aβ1-42 peptides (Mulder et al., 2016).

7.4 FUTURE PERSPECTIVES OF AD THERAPEUTICS

The challenge to investigate therapeutic strategies in neurodegeneration is the exact understanding of their neuropathological mechanisms. Cytokine expression is drastically altered in various neurodegenerative disorders (Bhaskar et al., 2014; Fillit et al., 1991). For

instance, up-regulated expression of TNF-α, TNFR1 and TRADD can be observed in AD (Fillit et al., 1991; Zhao et al., 2003). Neuroscientists have studied whether anti-TNF-α agents could be able to therapy neurodegenerative disorders, such as AD. However, anti-TNF-α reagents induced some side-effects in patients with multiple sclerosis (Sicotte and Voskuhl, 2001). Studies have shown that TNF-α interacting with two distinct receptors can perform the two opposite functions: TNF-α linking to TNFR1 induces neurodegeneration whereas TNF-α linking to TNFR2 rescues neurons from neurodegeneration (He et al., 2007, Williams et al., 2014). We thought that selective targeting TNFRs, inhibiting TNFR1 signalling and activating TNFR2 signalling, could effectively prevent neurodegenerative disorders. We indeed found that both TNFR2 agonist and TNFR1 antagonist prevent the neocortical denervation and improve the related memory deficits (Dong et al., 2016), as described in this thesis. This therapeutic strategy could improve the therapeutic deficits of anti-TNF-α agents because selective inhibiting TNFR1 and/or activating TNFR2 changed the rate of TNFR1/TNFR2 towards neuroprotective signalling and inhibiting TNFR1 rather than suppressed all TNF-α signalling. These suggest that TNFR1 targets or TNFR2 targets are worth to be considered to develop the therapeutic agents against AD. Apparently, the proof that TNFR1 antagonists and TNFR2 agonists prevented neurodegenerative disorders in an acute AD mouse model could be further provided from the chronic AD mouse model and the clinical trials with AD. The follow-up experiment derived from the thesis could focus on the therapeutic effecacy in chronic AD animal models. Before we test both compounds in chronic AD mouse models, a burning question should be resolved that both coumpounds must be redesigned to develop pharmacological drugs which can penetrate the blood-brain-barrier to respond the lesion regions in the brain of AD.

Furthermore, dependent on TNFR1-mediated signalling pathway, targeting a certain core mediator in apoptotic processes could provide a novel approach to treat AD. TNFR1 mediated cell death is mainly due to the core adaptor TNFR-associated death domain (TRADD) and Fas-associated death domain (FADD) activation. Therefore, that prevention of TREDD activation or FADD activation depresses TNFR1 death signalling could be an effective treatment for AD. An advantage of this strategy might be that just prevention of TREDD and FADD inhibits TNFR1-mediated death signalling but not interfere TNFR1-mediated other signalling pathways such as TNFR1-activated NF-κB signalling or TNFR2 neuroprotective effects. In future, some novel therapeutic strategies based on cytokine signalling pathways could be prevalent against neurodegenerative disorders.

In addition, accumulation of Aβ plaque peptides is responsible for the onset of AD. Although Aβ plaques are involved in the pathology of AD, Aβ plaques also seems to occur in non-diseased aging people (Dugger et al., 2014; Elobeid et al., 2016; Kovacs et al., 2013). This could result in the failure of anti-Aβ vaccines for AD. As we know, it seems that Aβ oligomers are mainly responsible for toxic stress in the brains in AD patients. Immunization against oligomeric Aβ might appear to be promising strategy. We indeed found that an anti-oligomeric Aβ vaccine prevent AD-related lesion (Chapter 6). However, immunization is a complicated approach that involves vaccine optimization and the possibility of side-effects. Consequently, immunization to treat AD could need more time and experiments to verify vaccine safty and effecacy.

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NEDERLANDSE SAMENVATTING

Neurodegeneratieve ziektes zijn gedefinieerd als genetische en sporadische condities, gekenmerkt door voortschrijdende degeneratie van het zenuwstelsel. Voorbeelden van neurodegeneratieve condities zijn de ziekte van Alzheimer, Parkinson, multiple sclerose (MS), amyotrofische laterale sclerose (ALS) en de ziekte van Huntington,maar ook bij een herseninfarct, hersenbloeding en traumatisch hersenletsel kan neurodegeneratie plaatsvinden, in het centrale of perifere zenuwstelsel. In deze thesis hebben we potentiele therapeutische strategieën tegen neurodegeneratie onderzocht, in het Nucleus Basalis Magnocellularis (NBM) lesie model. NBM lesies resulteren in het verlies van cholinerge neuronale projecties in de cerebrale cortex, en kunnen dienen als een acuut model voor de ziekte van Alzheimer.

Tumor necrosis factor alfa (TNF-α) is voorgesteld als een belangrijke speler in neurodegeneratie, zoals in de ziekte van Alzheimer, MS en herseninfarct. Terwijl TNF-α via het activeren van TNF receptor 1 voornamelijk schadelijke effecten op hersencellen heeft, induceert TNF-α via TNFR receptor 2 juist neuro-protectieve effecten. Gebaseerd op de tegenovergestelde effecten van TNF-α via TNF receptor 1 en TNF receptor 2, hebben we de effecten van een humaan-specifieke antagonist van TNF receptor 1 (ATROSAB) en specifieke agonisten van TNF receptor 2 (TNC-scTNF en EHD2-scTNF) onderzocht in het NBM lesie muismodel. Omdat deze stoffen niet werken op muis TNF receptoren, hebben we gehumaniseerde TNF receptor knock-in muizen ontwikkeld, waarin de extracellulaire domeinen van de endogene muis TNF receptoren zijn vervangen door hun humane tegenhangers. In deze muizen vonden we dat beide TNF receptor-modulatoren in staat waren neuronale schade en ontstekingen als gevolg van de NBM lesie significant te verminderen, en cognitieve achteruitgang tegen te gaan. Een belangrijke bevinding hierbij was dat activatie van TNF receptor 2 essentieel is voor neuroprotectie. In een andere studie vonden we dat immunisatie van muizen met cyclopeptide-conjugaten, afgeleid van

oligomeer Aβ, beschermend werkte tegen oligomeer Aβ-geïnduceerde NBM lesies in muizen. In conclusie hebben we in deze thesis twee potentiele therapeutische interventies onderzocht voor de behandeling van de ziekte van Alzheimer.

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Yun Dong

LIST OF PUBLICATIONS

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CURRICULUM VITAE

Yun Dong was born on 13 May 1982 in Heilongjiang, China. Yun finished secondary education in Kedong High School in 2003. Thereafter she studied biological technology at Henan University of Science and Technology in Luoyang (China) in 2003. Since then, she commenced her interest in science. After successful graduation in 2006, Yun studied biology at Nanyang Normal University, with cell biology and biochemistry as major subjects. She got the diploma for her secondary bachelor's degree from Nan yang Mormal Unversity in 2008. After successful completion of her bachelor's degree, she followed a master's degree at Lanzhou University in Lanzhou (China). Her project was based on the investigation in the risks of mobile radiation for eye. During her master's program, she gained experience in cell culture, histochemistry stainings, western blot, and analytical techniques and animal experiments. An international conference at Lanzhou University influenced her carer direction, in which a professor showed her studies on neurodegenerative disease. Since that time, Yun decided to study in neuroscience. She completed her master program under the supervision of Prof. dr. Jianlin Wang in 2011. She went abroad to do her PhD with support from Chinese Scholarship Council. She started a 4-year PhD program in the group of Prof. Dr. U.L.M. Eisel in the Department of Molecular Neurobiology of the University of Groningen, The Netherlands. This project focused on the investigation in different therapeutic approachess in the NBM lesion mouse models. The completion of this project led to the present dissertation titled 'therapeutic trategies for neurodegenerative disorders'. The work described in this thesis was supervised by Prof. dr. U.L.M. Eisel and Prof. dr. E.A. van der Zee, Department of Molecular Neurobiology, Groningen, The Netherlands. Since 31th of May 2016 she works as an assistant investigator at the Brain Cognition and Brain Disease Institute for Collaboration Research of SIAT at CAS and the McGovern Institute at MIT, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, China.